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**SMALL ARTERY
DYSFUNCTION:
FOCUS ON
PREECLAMPSIA
AND END-STAGE RENAL
DISEASE**

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To my family

ABSTRACT

Background: End-stage renal disease (ESRD) and preeclampsia (PE) are associated with high risk of cardiovascular events and characterized by endothelial dysfunction. Resistance arteries are actively involved in the control of blood pressure and blood flow to the target organs. The expansion of the current knowledge specific to the structure and function of resistance arteries in subjects with increased risk of cardiovascular events is of importance for clinical practice.

Overall aim: To investigate structure and function of resistance arteries isolated from ESRD patients, women with PE and women at reproductive age with a history of early-onset PE.

Results:

Study I. Reduced flow- and acetylcholine-induced dilatations, but preserve response to NO donor were found in arteries from the ESRD group vs. controls. Deficiency of NO was evident in flow-induced response. Distensibility was reduced in the ESRD group, but vascular structure, myogenic tone and sensitivity to vasoconstrictors remained unchanged. Increased ADMA levels and enhanced expression of nitrotyrosine were found in arteries from the ESRD group. Exclusion of ESRD patients with diabetes and/or cardiovascular diseases from analyses had no influence on the main findings.

Studies II-III. Dilatation to bradykinin (BK) was reduced in myometrial but not in subcutaneous arteries from pregnant women with PE vs. controls. In PE, endothelium-derived hyperpolarizing factor (EDHF)-type responses were impaired in both types of arteries. The contribution of myoendothelial gap junctions (MEGJs), implicated as a common pathway of EDHF-type responses in arteries from normal pregnant women, became reduced in subcutaneous and particularly diminutive in myometrial arteries from women with PE. The reduced contribution of MEGJs in PE was partly compensated by H₂O₂ alone (myometrial) or in combination with cytochrome P450 metabolites of arachidonic acid (subcutaneous arteries).

Study IV. Reduced dilatation to flow due to the lack of NO contribution, increased myogenic tone, higher sensitivity to norepinephrine and reduced distensibility were observed in arteries from women with a history of early-onset PE vs. controls. Responses to BK and NO donor were similar between the groups. Basal tone and arterial structure were preserved in women with a history of PE.

Conclusions: Uraemia primarily targets endothelial function and elastic properties of resistance arteries. The lack of NO contribution to flow-induced dilatation together with enhanced circulating levels of ADMA and enhanced nitrotyrosine staining in the vascular wall support the critical role of NO deficiency in resistance arteries from ESRD patients.

In PE endothelial function of myometrial arteries is primarily targeted. This could significantly contribute to the impaired uteroplacental blood flow. EDHF-type responses through MEGJs are the major compromised pathway of endothelium-dependent dilatation in both myometrial and subcutaneous arteries in PE. The attenuated role of MEGJs in PE is partly compensated through the contribution of H₂O₂ or other endothelium-derived factors.

Functional alterations in subcutaneous arteries from women with a history of early-onset PE might create prerequisites for the increased peripheral resistance with following impact on the long-term cardiovascular health.

Key words: Resistance arteries, preeclampsia, end-stage renal disease, endothelium, nitric oxide, endothelium-derived hyperpolarizing factor, flow-induced dilatation, myogenic tone, distensibility.

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

18- α GA	18- α -glycyrrhetic acid
AA	Arachidonic Acid
ADMA	Asymmetric dimethyl-L-arginine
Akt	Protein kinase B
Ang II	Angiotensin II
AT1	Angiotensin II receptor type 1
AT1-AA	Angiotensin II type I receptor agonistic autoantibody
ATP	Adenosine triphosphate
BH ₄	Tetrahydrobiopterin
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CI	Confidence interval
CKD	Chronic kidney disease
COX	Cyclooxygenase
CSA	Cross-sectional area
CVD	Cardiovascular diseases
Cx	Connexin
CYP450	Cytochrome P450
ECs	Endothelial cells
EDHF	Endothelium-derived hyperpolarizing factor
EETs	Epoxyeicosatrienoic acids
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
ESRD	End-stage renal disease
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
H ₂ O ₂	Hydrogen peroxide
HbA1c	Glycated hemoglobin
HELLP	Hemolysis, elevated liver enzyme levels, and low platelet count
HDL	High-density lipoprotein
HOMA	Homeostatic model assessment
hsCRP	High sensitivity C-reactive protein
HSP90	Heat-shock protein 90
ICH	Immunohistochemistry
IEL	Internal elastic lamina
IgG	Immunoglobulin G
IL	Interleukin
Indo	Indomethacin
IUGR	Intrauterine growth restriction
KPSS	High potassium physiological salt solution
LDL	Low-density lipoprotein

L-NAME	<i>N</i> ⁰ -nitro-L-arginine-methyl ester
MEGJs	Myoendothelial gap junctions
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Norepinephrine
NGS	Normal goat serum
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PE	Preeclampsia
pEC ₅₀	Negative log concentration (mol/l) required to achieve 50% of the maximum response to the agonist
PGI ₂	Prostaglandin I ₂ or prostacyclin
PIGF	Placental growth factor
PSS	Physiological salt solution
RR	Relative risk
RUPP	Reduced uterine perfusion pressure
sEng	Soluble endoglin
SGA	Small for gestational age
sFlt-1	Soluble fms-like tyrosine kinase-1
sGC	Soluble guanylyl cyclase
SMCs	Smooth muscle cells
SNP	Sodium nitroprusside
TGF-β	Transforming growth factor β
TNF-α	Tumor necrosis factor-alpha
tPA	Tissue plasminogen activator
TX A ₂	Thromboxane A ₂
VEGF	Vascular endothelial growth factor
VP	Vasopressin
vs.	Versus
vWF	von Willebrand factor

1 INTRODUCTION

Small arteries with internal diameter within range from 0.1 to 0.3 mm and arterioles (diameter of $<0.1\text{mm}$) are the major site of resistance to blood flow and as such they contribute considerably to the control of peripheral resistance, blood pressure and blood flow. The disturbances in these parameters of cardiovascular function represent a background for the development of a number of cardiovascular diseases (CVD). Thus, the elucidation of functional and morphological abnormalities that take place at the level of resistance circulation may provide means for primary and secondary prevention of cardiovascular complications in the wide range of different patients.

A tremendous importance of small arteries in the maintenance of cardiovascular function explains a considerable interest of biomedical research to the structural and functional characteristics of these arteries in health and disease. Moreover, a previous approach of extrapolation of the knowledge obtained on large arteries to the level of microcirculation could not be considered as appropriate. Indeed, current data clearly indicates on morphological and functional differences between small and large arteries. For example, several mechanisms involved in the control of vasomotor tone at the level of small arteries (e.g. myogenic autoregulation, flow-induced and metabolic dilatation, contribution of endothelium-derived hyperpolarizing factor (EDHF) and gap junctions to the endothelium-dependent function, sympathetic constriction, etc.) are very poor, if at all, represented at the level of conduit arteries. Therefore, the extension of the knowledge peculiar to morphology and function of small arteries in health and disease is of importance for clinical practice.

The general aim of this thesis is to expand the current knowledge about the structure and vasomotor tone control of small arteries in patients with end-stage renal disease (ESRD), pregnancy-specific disorder preeclampsia (PE) and in women at reproductive age with a history of PE.

1.1 SMALL ARTERY STRUCTURE

As in any other blood vessels, the vascular wall of small arteries consists of three layers (Mulvany, 1990). Each layer has certain influence on the vasomotor tone. Tunica intima is the inner layer, which composed of endothelial cells (ECs) oriented longitudinally to sense shear stress forces created by blood flow. Despite the endothelium has the only one layer of ECs, its role in the control of vasomotor tone through the release of vasoactive substances is of vast importance. This will be described below in the details.

The endothelium is separated by the internal elastic lamina (IEL) from the underlying layer i.e. tunica media. IEL of small arteries is fenestrated and protrusions from ECs and smooth muscle cells (SMCs) can reach each other via formation of myoendothelial gap junctions (MEGJs), which play an important role in numerous aspects of vascular function and pathology (Dhein, 2004).

The thickest layer of the vascular wall, tunica media, containing SMCs oriented perpendicularly to the lumen of the artery and therefore can develop circumferential force. External elastic lamina separates tunica media and adventitia. The outer adventitial layer includes perivascular sympathetic nerves, extracellular matrix and

fibroblasts. Recent data indicate that adventitia contains proteins that could play an important role in vascular smooth muscle function as well (Auger et al., 2007).

The structure of small arteries is not static and under influence of the hemodynamic conditions. Changes in the wall/lumen ratio and cross-section area reflect structural alterations and indicate a remodelling process.

1.2 THE ROLE OF SMALL ARTERIES

In contrast to the conduit arteries, the vascular wall of small arteries contains more smooth muscles than elastic materials reflecting the specificity of their functional properties. Small arteries can easily adapt their diameter by constriction or dilatation in accordance with metabolic needs of the supplied tissue or organs.

In accordance with Poiseuille's law, the resistance can be expressed as:

$$R=8\eta L/\pi r^4,$$

where η is viscosity of fluid, L – length, and r – radius of the vessel. Because of the fourth power of the radius, small changes in vascular diameter will have significant effects on resistance and blood flow. This in turn will directly influence blood pressure, since

$$P=R\times Q,$$

where P is pressure, R – resistance, and Q – blood flow.

Hereby, small arteries, because of their intrinsic ability to change the diameter, play an important role in the regulation of blood flow to the target organs and have a pivotal role in the control of systemic blood pressure (Christensen and Mulvany, 2001).

1.3 THE VASCULAR TONE REGULATION

The diameter of small arteries is under central (neuro-humoral) and local regulations. The relative contribution of each might be different depending from the vascular beds and physiological conditions. For example, the hormonal regulation has minor influence on the arterial diameter in the healthy individuals under normal conditions, but it becomes predominant under physical activity or in some pathophysiological situations.

Mechanisms of local vascular control involve myogenic activity and local humoral factors. Myogenic regulation is based on an intrinsic mode of control of vascular reactivity i.e. without direct effects of nerves and hormones. Stretch, mediated by perfusion pressure, triggers the mechanism of myogenic constriction. Humoral factors, which include metabolites, hormones, and mediators, affect significantly vascular tone. For example, any chemical alterations in the interstitial fluid mediated by high metabolic rate (e.g., the low PO₂, pH, and adenosine triphosphate (ATP) levels as well as the high levels of PCO₂, adenosine, and K⁺) will challenge relaxation and increase blood flow.

1.3.1 Endothelium-dependent regulation of the vasomotor tone

Strategically located between the blood and vascular wall, the endothelium is a major player in the control of blood fluidity, platelet aggregation, permeability, and vascular tone regulation. Furthermore, the endothelium is the largest endocrine organ in the

body and it has an important role in the regulation of inflammation, immunological responses, and angiogenesis.

ECs control the tone of the underlying vascular SMCs by releasing relaxing and contracting factors and by interacting directly with the vascular SMCs through the MEGJs (Feletou M, 2008). Vasodilator capacity of the endothelium is accounted by its ability to release primarily three powerful factors, namely nitric oxide (NO), EDHF and prostacyclin (PGI₂), whereas endothelin-1 (ET-1) and thromboxane A₂ (TX A₂) are the main endothelium-derived vasoconstrictors.

NO, a short-lived free radical gas with a high biological activity, is produced by L-arginine-endothelial nitric oxide synthase (eNOS) system in the presence of molecular oxygen (Palmer et al., 1987). The eNOS is expressed constantly and associated with caveolin-1, which inhibits eNOS activity (Dudzinski and Michel, 2007). eNOS-calcium/calmodulin interaction is required for its activation. Heat-shock protein 90 (HSP-90) acts as a chaperone and increases eNOS activity by enhancing the affinity of eNOS for calmodulin and facilitates the interaction with Akt kinase (Garcia-Cardena et al., 1998). Several co-factors are required including nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin (BH₄) in order to catalyze this reaction (Forstermann et al., 1994). NO activates soluble guanylyl cyclase (sGC) of SMCs, with following increase in intracellular cyclic guanosine monophosphate (cGMP) and activation of protein kinase G leading to reduced intracellular calcium and relaxation (Hobbs and Ignarro, 1996).

PGI₂ – a cyclooxygenase (COX)-dependent metabolite of arachidonic acid (AA) (Dusting GJ, 1977). PGI₂ elicits relaxation through binding to specific cell-surface PGI₂ receptors resulting in G-protein mediated activation of adenylate cyclase and formation of cAMP (cyclic adenosine monophosphate). This phosphorylates protein kinase A and results in the reduction of calcium in SMCs and vasodilatation (Mitchell et al., 2008).

Hyperpolarization is a mechanism behind of the third pathway of endothelium-dependent relaxation, which is known as EDHF type response. This pathway of endothelial control of vascular tone is considered as a signal generated and released by hyperpolarized ECs. Endothelium-derived hyperpolarizing signal can activate K⁺ channels and causes hyperpolarization of the underlying SMCs. In turn, hyperpolarization of vascular SMCs elicits the closure of voltage-gated calcium channels (Edwards et al., 2010) followed by decrease in intracellular calcium concentration and subsequent relaxation. Currently, there are numerous evidences for heterogeneous nature of EDHF. Structurally very different factors including cytochrome P450 (CYP450) products of AA (Rubanyi GM, 1987; Campbell and Fleming, 2010), hydrogen peroxide (H₂O₂) (Shimokawa and Morikawa, 2005), C-type natriuretic peptide (Chauhan et al., 2003) and potassium ions released by hyperpolarized ECs (Edwards et al., 1998) have been introduced as potential candidates for EDHF. Furthermore, ECs hyperpolarisation could be also transmitted to SMCs through MEGJs (Chaytor et al., 1998) that are clusters of intercellular channels formed by membrane-located connexin (Cx) proteins. There is a number of excellent reviews summarizing current data about potential candidates for the role of EDHF (McGuire JJ, 2001; Félétou and Vanhoutte, 2006; Luksha et al., 2009; Edwards and Félétou et al., 2010).

Hereby, the endothelium is actively involved in the regulation of vascular tone via release of vasoactive compounds. Some of them are released continuously, whereas others after stimulation with either pharmacological (endothelium-dependent agonists i.e. acetylcholine (ACh), bradykinin (BK), substance P) or physical/mechanical (flow-induced shear stress) stimuli. Physiologically, ECs maintain a relaxed vascular tone and low levels of oxidative stress. Therefore, healthy endothelium, as defined in terms of the vasodilator response to endothelium-dependent agonists and blood flow, is an important indicator of cardiovascular health. The endothelium is not only largest endocrine/paracrine organ in the human body, but also the target organ for many active substances. Endothelial dysfunction is defined as reversible alterations of endothelial function resulting from the misbalance between endothelium-derived vasodilators and vasoconstrictors. It is now widely appreciated that endothelial dysfunction is the initial pathophysiological step in progression of vascular damage that leads to CVD (Katakam et al., 1998; Prasad et al., 2003; Szűcs et al., 2007).

1.3.2 Hemodynamic forces in the control of vascular tone

Mechanical forces associated with blood flow play an important role in the acute control of vascular tone. Forces acting on vascular wall due to blood flow can be divided into two principal vectors. One is parallel to the wall to create a frictional force, shear stress, at the surface of the endothelium, and the other acts perpendicular to the wall and represents blood pressure (Davies, 1995). Whereas transmural pressure is transferred to all vascular wall layers (intima, media and adventitia), shear stress is applied only on the inner EC layer.

1.3.2.1 Flow-induced responses

The physiological relevance of flow-induced relaxation firstly refers to the regulation of arterial diameter. In addition, physiological shear stress, induced by laminar blood flow, may act as an atheroprotective factor (Davies, 1995) and promote anticoagulation through the inhibition of platelet aggregation (Diamond et al., 1989), leukocyte adhesion and SMCs proliferation (Berk et al., 2001).

Significance of blood flow, as an important hemodynamic force in the control of the vasomotor tone, was reported for the first time by Schretzenmayr in 1933. However, the mechanism of flow-induced relaxation is still under investigations with an especial interest into the molecular mechanisms of transduction of shear stress forces into the cellular response.

Shear stress stimulates ECs and initiates the mechanotransduction process. Mechanotransduction serves as concerting action, which is initiated by mechanical deformation of ECs with following intracellular transmission of stress and conversion of mechanical force into chemical activity. Finally, downstream biochemical signaling leads to physiological responses (Li et al., 2005; Lehoux et al., 2006; Davies, 2009).

Shear stress is determined by three variables: blood velocity, blood viscosity, and vascular diameter. When the diameter is reduced, or when blood velocity or viscosity is elevated, the shear stress imposed on the endothelium is increased. The general approach of flow-induced regulation serves as the normal positive feedback mechanism of greater blood flow causing greater shear stress, which in turn has a remodelling effect to normalize shear stress by relaxation, and thereby allowing greater blood flow.

The role of the endothelium-derived factors in flow-induced responses was established 50 years after discovery of flow-dependent vasoregulation (Hull et al., 1986). Now it is generally accepted that flow-induced relaxation is an endothelium-dependent process. Distal arteries relax in response to tissue metabolites, whereas in proximal arteries increased flow induces secondary relaxation through endothelium-dependent mechanisms.

Current dogma is that endothelium-derived NO plays a key role in the regulation of flow-mediated dilatation in healthy condition (Joannides et al., 1995; Shioda et al., 1996). Indeed, after application of shear stress, endothelial NO production is enhanced twofold to fourfold over basal values and is maintained as long as the stimulus is applied (Fleming et al., 2005).

The activation of eNOS by shear stress or with agonist differs in respect to their sensitivity to the concentration of extracellular Ca^{2+} . Shear stress induced responses are relatively insensitive to changes in intracellular Ca^{2+} whereas agonist-induced responses highly Ca^{2+} -sensitive (Fleming et al., 2001). Indeed, flow-induced NO production is a biphasic process. The first phase is Ca^{2+} dependent process when calmodulin binds to eNOS with following rapid dissociation from caveolin and subsequent association with HSP-90. This favours to eNOS phosphorylation on most known serine residue (Ser 1177) by protein kinase A (Fleming, 2010). Phosphorylated eNOS becomes more sensitive to Ca^{2+} and, at the second phase, eNOS can be activated at resting Ca^{2+} levels. This is so-called “calcium-independent” activation of eNOS in response to shear stress, which results in maintained production of NO (Balligand et al., 2009).

However, flow-induced responses persist in mice lacking eNOS (Huang et al., 2001) and multiple mediators are further implicated. PGI_2 (Koller et al., 1995) and cytochrome P450 metabolites of AA (Miura et al., 2001; Sun D, 2007) can contribute to the flow-induced dilatation as well. Recently H_2O_2 has been introduced as a necessary component of flow-induced vasodilatation (Drouin and Thorin, 2009; Kang et al., 2009) or as an alternative mediator replacing NO-mediated mechanism in CVD (Phillips et al., 2007; Liu et al., 2011). Moreover, predominance of EDHF-type regulation of flow-induced dilatation has been found in rats born growth restricted (Morton et al., 2011).

Thus, flow-induced vasodilatation is an integrated response involving the release of several endothelium-derived vasodilator autacoids. Although NO is generally described as the primary endothelium-derived relaxing factor, the contribution of other endothelium-derived vasodilators should be taken in consideration as well.

1.3.2.2 Response to pressure

Arteries are constantly exposed to blood pressure and cyclic stretching. In response to these forces arteries develop the myogenic response, a state of intrinsic constriction of SMCs, which is independent of neurohumoral and endothelial influences (Davis and Hill, 1999) (Khavandi et al., 2009). Myogenic constriction is an important component of basal vascular tone (Hill and Davis, 2007). Myogenic constriction can be considered as a background tone preparing the contractile apparatus to contract more or less in response to applied stimuli providing the local regulation of blood flow and pressure in rapid and efficient manner.

Osol *et al* have described a three-phase model of *in vitro* arterial myogenic behavior. The first phase is characterized by development of tone (myogenic tone; 40–60 mmHg). The second phase, myogenic reactivity (60 and 140 mmHg), reflects the response to changes in transmural pressure. Elevated transmural pressure leads to myogenic constriction, whereas reduced pressure leads to myogenic dilatation. The third phase occurs when the vascular wall of the artery is unable to maintain the constriction against transmural pressure (above 140 mmHg) known as forced dilatation, a complete loss of myogenic tone (Osol *et al.*, 2002). Only small arteries with a relaxed internal diameter <300µm have the property to develop myogenic response (Khavandi and Greenstein *et al.*, 2009). Larger and very small arteries possess a relatively weak myogenic response. Furthermore, the strength of myogenic response varies in diverse vascular beds of animals and human (Davis and Sikes, 1990; Osol *et al.*, 1991; Watanabe *et al.*, 1993; Liao and Kuo, 1997; Miller *et al.*, 1997).

The myogenic response first was described by Bayliss at the beginning of the last century. Despite intensive investigation for more than 100 years and its physiological and pathophysiological significance, the upstream mechanisms that translate pressure changes to vascular SMCs contractions are not entirely understood. Currently the following sequence of events is considered to be involved in myogenic response. Increased intraluminal pressure is conveyed through the ion channels, or membrane lipids bilayer itself, or through extracellular matrix-integrin complex. Activation of these pathways leads to the opening of non-selective cation channels, membrane depolarization and the opening of voltage-gated, L-type Ca^{2+} channels. The elevated level of Ca^{2+} results in Ca^{2+} /calmodulin-dependent activation of myosin light chain kinase, phosphorylation of the 20-kDa myosin regulatory light chains and leads to contractile interaction of actin and myosin (Zou *et al.*, 2000; Hill *et al.*, 2006; Hill *et al.*, 2009). More likely, these pathways are activated in parallel and interact with each other.

The outcome of pressure-induced responses depends from the duration of exposure of vascular wall to the increased intraluminal pressure. After prolong exposure, an acute contractile state is eventually replaced by structural alterations within the vascular wall known as pressure related remodelling (Mulvany, 2011). Eutrophic inward remodeling is characterized by reduction in internal diameter with an increase in wall thickness and considered as a physiological response to elevated blood pressure. This type of remodelling involves reorganization of the existing elements of the vascular wall and may be considered as an intermediated response. Hypertrophic remodelling occurs when the ability of artery for autoregulation is depleted and artery is unable to resist the increased wall tension any longer. Hypertrophic remodelling is characterized by an increase amount of vascular wall tissue, due to SMCs hypertrophy or hyperplasia, and indicates a functional disorder and imminent target organ damage.

The physiological relevance of myogenic constriction refers to control the microcirculation of target organs, providing a protection of vulnerable organs such as brain, heart and kidney from pressure related damage. Therefore, the impact of abnormal myogenic responses in the pathogenesis of CVD and protection against hypertension-induced organ damage is widely disputed (Davis and Hill, 1999; Hughes and Bund, 2002).

To sum up, two mechanical stimuli, flow and pressure, play an important role in the local regulation of vascular tone of small arteries. These two most relevant

physiological stimuli are applicable for investigation of resistance artery function *ex vivo* in the pressure myography system.

1.4 VASCULAR STRUCTURE AND FUNCTION IN KIDNEY FAILURE

Kidney failure is a worldwide public health problem, with increasing incidence and prevalence, high costs, and poor outcomes (Eknoyan et al., 2004). A progressive loss in renal function leads to ESRD, end point of chronic kidney disease (CKD) with kidney function less than 15% of normal. Clinical evidences show that patients with renal failure have a higher prevalence of systemic vascular disease (Agodoa, 2000; Mailloux, 2001; Kalra et al., 2005).

Despite the rapid progress in dialysis treatment, life expectancy in ESRD patients is severely reduced and the major cause of mortality is attributed to CVD (Foley et al., 1998; Go et al., 2004). Moreover, all existing treatment strategies aimed at reduction of CVD such as increased dialysis dose, homocysteine-lowering therapy, intensified nutrition, lipid-lowering, antihypertensive treatment, and normalization of haemoglobin have not shown a survival benefit (Nanayakkara, 2010). This fact may indicate that mechanisms underlying pathophysiology of cardiovascular complications of CKD are complex and still far from clear.

Hypertension is diagnosed in the majority of ESRD patients (Agarwal et al., 2003). In light of this fact, hypertension can be considered as a major component of the relationships between CKD and CVD (Ravera et al., 2006). Altered myogenic activity and endothelial dysfunction are closely related to hypertension and cardiovascular events in patients with renal failure. Hereby, investigation of structure and function of small arteries, which are responsible for the regulation of blood pressure, is of importance. Expanded knowledge might provide a new insight into the mechanisms of cardiovascular pathophysiology in ESRD.

Until now most of the studies of vascular structure and function in kidney disease have been conducted on human large arteries. Increased carotid arterial intima-media thickness and stiffness have been reported in patients with kidney failure (Rahn KH, 2000; Preston et al., 2005; Allison, 2010; Makita et al., 2010). Thickening of the intima-media-complex is an early sign of atherosclerosis in big and medium-sized arteries. It reduces the distensibility of the arteries during systole. Vascular structural alterations tightly correlate with functional abnormalities (Juonala et al., 2004). Indeed, endothelial dysfunction is a common phenomenon in patients with kidney disease (Passauer et al., 2000; Annuk, 2005; Sahin et al., 2007; Verbeke et al., 2007). Moreover, endothelial dysfunction has been detected at an early phase of renal injury (Thambyrajah, 2000; Kuczmarski, 2011). This can indicate that endothelial dysfunction related to renal failure can promote development of CVD and contribute to the progression of kidney failure.

Chronic renal failure *per se* is considered as a state of NO deficiency (Blum, 1998; Baylis, 2008). Investigation of mechanisms behind of endothelial dysfunction in large arteries has revealed impairment of both NO-and EDHF-mediated pathways depending on the vascular beds. *In vivo* studies have provided an indirect evidence of impaired contribution of NO to flow-induced responses in ESRD patients (Leone et al., 1992; Yilmaz, 2005).

The mechanisms of endothelial dysfunction in renal failure have been studied more in details on subtotaly nephrectomized rats. A lack of NO contribution to flow-induced relaxation has been observed on isolated femoral arteries, suggesting that other endothelium-derived mediators might be involved (Savage et al., 2001). ACh-induced relaxation of superior mesenteric arteries mediated by both NO and EDHF has been attenuated (Kimura, 1999). Severe impairment of EDHF-mediated relaxation has been shown in carotid arteries (Kohler et al., 2005).

These findings on large arteries leave unaddressed the issue of whether and/or to what extent functional and structural abnormalities may occur concurrently in the resistance circulation. Unfortunately, the structure of resistance arteries has not been properly investigated. A few studies on animals have suggested that uraemic environment affects the vascular structure, pointing towards remodelling process in small arteries. A substantial increase in the wall thickness of small intramyocardial arteries (Nabokov et al., 1999), increased wall/lumen ratio and decreased lumen diameter have been reported in cremaster and mesenteric arteries, although no significant changes have been observed in cerebral arteries of subtotaly nephrectomized rats, compared to normotensive controls (New et al., 2004). However, an early investigation has suggested that the advanced human uraemia state was not associated with changes in vascular morphology in subcutaneous arteries (Aalkjaer, 1986).

Also there are limited and controversial data in respect to functional properties of resistance arteries in uraemic environment. Since myogenic constriction depends on intraluminal pressure, it is reasonable to assume that hypertensive uraemic environment would increase the myogenic constriction and contribute to the increased peripheral resistance. Unexpectedly, no changes in myogenic constriction in skeletal muscle arteries (New et al., 2003) or even reduction in myogenic response in cerebral (New et al., 2003) and in small mesenteric arteries (Vettoretti, 2006) have been reported. Unfortunately, so far only data obtained from animal models are available in regards to myogenic response in uraemia.

It is important to specify, that in spite of clinical evidences of impaired vascular function in renal failure there is still a lack of knowledge regarding to the state of endothelial function of small arteries. So far only one *ex vivo* study has provided evidence of disturbances of endothelial function in human subcutaneous resistance arteries. Reduced response to ACh but preserved response to NO donor have been reported in patients with chronic renal failure (Morris et al., 2001). The mechanism behind endothelial dysfunction has been only speculated but not addressed. Inconsistent results have been reported using *in vivo* investigation of skin microcirculation. Stewart *et al.* have shown impaired endothelial function in ESRD patients without CVD and diabetes mellitus (Stewart et al., 2004). In contrast, normotensive patients with chronic renal failure had preserved endothelium-dependent and -independent responses (Cupisti, 2000).

Too little is known about the mechanisms of endothelial injury in kidney disease at the level of resistance arteries. Measurement of forearm blood flow in hemodialysis patients has provided an evidence of reduced stimulation of NO in agonist-induced endothelium-dependent vasodilatation (Passauer et al., 2005) and suggested that CYP 2C9-derived products did not participate in the regulation of arteriolar tone (Passauer et al., 2005). Also impaired flow-induced dilatation due to the lack of NO has been observed in gracilis skeletal muscle arterioles in rats after subtotal nephrectomy (Bagi, 2003).

Thus, the expected morphological and functional alterations present in the small arteries of CKD patients are largely uncharacterized and unexplored. Since correction of endothelial dysfunction in uraemic environment can be an attractive topic for pharmacological approaches (Rabelink and Koomans, 1997), comprehensive understanding of the pathophysiology of resistance vasculature in CKD might lead to the development of new approaches to prevent or ameliorate the microvascular abnormalities.

1.5 PREECLAMPSIA: PATHOGENESIS AND VASCULAR TARGETS

1.5.1 Pregnancy-induced cardiovascular alterations

Normal pregnancy is accompanied by tremendous changes in the maternal cardiovascular system in order to provide an adjustment of blood and nutrient supply to constantly increasing demands of the growing fetus, placenta and uterus. The cardiovascular pregnancy-induced alterations include expansion of blood volume, increase of cardiac output, heart rate, and reduction of blood pressure and systemic vascular resistance. Indeed, blood pressure falls until 20 weeks of gestation and returns to the initial level at term. Moreover, elevated endothelium-dependent relaxation to agonists and flow with reduced vasoconstriction to angiotensin II (Ang II) and norepinephrine (NE) have been shown in normal pregnancy (Gant et al., 1973; Nisell et al., 1985; Knock and Poston, 1996). Thus, the physiological adaptation to normal pregnancy can be considered as a “stress test” for cardiovascular system in particular (Williams, 2003). The vast majority of women go through this test without any complications, although some develop PE.

1.5.2 Preeclampsia: definition and clinical syndrome

Preeclampsia is a disorder specific to human pregnancy and diagnosed in the presence of hypertension (i.e. blood pressure $\geq 140/90$ mmHg after 20th week of gestation in previously normotensive women) accompanied by proteinuria (≥ 300 mg in 24-h urine sample) (Sibai et al., 2005). Up to 5-7% of pregnancies worldwide are complicated by PE (Walker, 2000; Sibai, 2003) and continues to be one of the leading causes of maternal and fetal morbidity and mortality with 15-20% of the total maternal mortality in developed countries (Sibai and Dekker et al., 2005), whereas in developing countries maternal mortality is more common (up to 3 times higher), accounting for 50000 deaths yearly (Duley, 1992).

The most common form of PE is late-onset PE (>34 weeks) with relatively slow progression and without many subjective symptoms or related pathologies. Early-onset PE (i.e. <34 weeks) is usually more severe form causing maternal and fetal morbidity with increased risk for intrauterine growth restriction (IUGR). This form of PE, especially if recurrent in subsequent pregnancies, may include impairments in liver and kidney functions, coagulation and central nervous system and be related to maternal chronic diseases such as thrombophilia and renal failure (Sibai and Dekker et al., 2005).

The majority of clinical symptoms usually disappear already after delivery. Therefore, it is difficult to distinguish a link between complicated pregnancy and cardiovascular health in later life.

1.5.3 Pathogenesis of preeclampsia

Despite of intensive research, the pathogenesis of PE is still not completely clear. Nowadays the most accepted is a two-stage theory of PE pathogenesis (Figure 1.1), which considering the presence of reduced placental perfusion as the first stage, and following maternal response to abnormal placentation as the second stage (Mutter and Karumanchi; Ness and Roberts, 1996; Pijnenborg et al., 2006). Indeed, in the majority of cases PE is characterized by abnormal placentation. It is generally assumed that poor placental perfusion in PE is a result of shallow cytotrophoblast invasion and the failure of maternal uterine spiral artery to undergo normal remodelling. In healthy pregnancy the remodelling of spiral arteries results in increased vascular luminal diameter up to four times and vascular wall sheds the smooth muscle and the inner elastic lamina (Whitley and Cartwright, 2010). The absence of vascular wall components tenders these vessels unable to respond to vasoactive stimuli (Roberts and Gammill, 2005). So in general, remodelling is modification of vessels to flaccid tubes, which provide a low resistance and high blood flow to meet the oxygen and nutrients needs for the developing fetus/placenta/uterus.

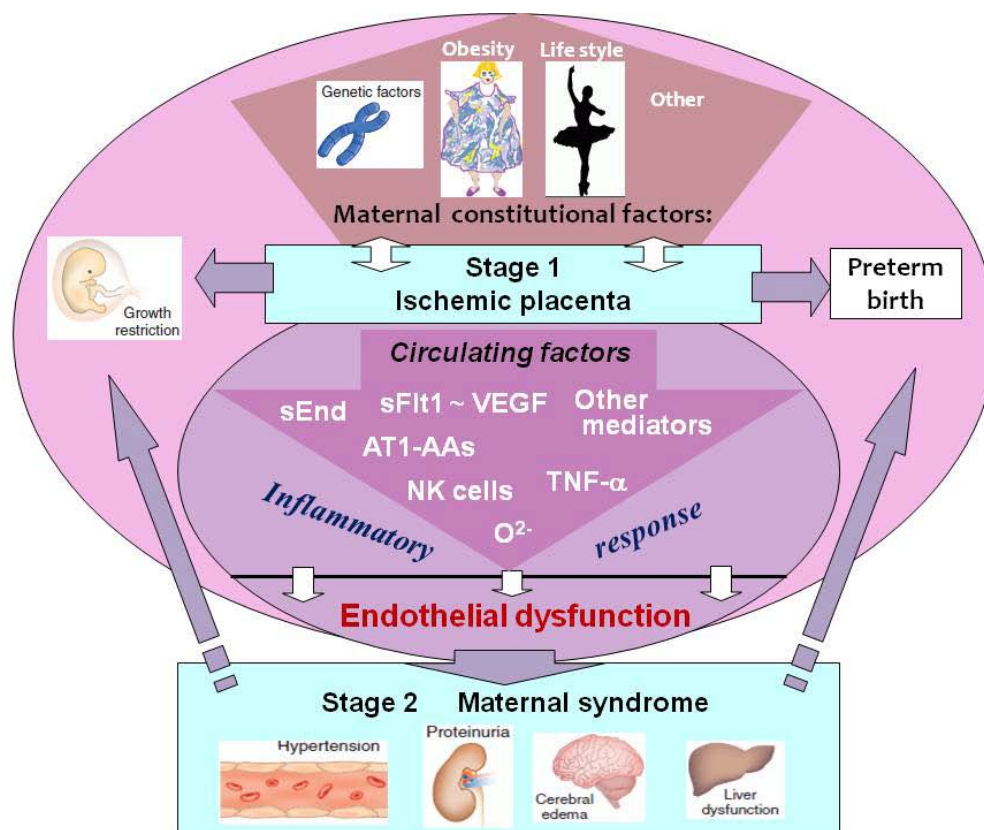


Figure 1.1. Two stage model of the pathophysiological changes associated with preeclampsia.

This process is aberrant in PE and, as a consequence, the poor placental perfusion occurs. The increased resistance in arteries supplying the intervillous space of women with PE was estimated by Doppler velocimetry assessment (Papageorgiou et al., 2002). The development of severe PE in women suffering from molar pregnancies confirms importance of the placenta rather than fetus in the pathogenesis of PE (Soto-Wright et

al., 1995). However, decreased placental perfusion might be necessary condition but it is not always sufficient for the development of PE. In clinical practice there are cases of PE without alterations in the placental perfusion (Roberts and Lain, 2002). Moreover, poor placentation is not a prerogative of only PE. Failure of normal placentation has been found in one-third of pregnancies ended by spontaneous preterm delivery (Arias et al., 1993), in non-hypertensive cases of IUGR (Khong et al., 1986), non-proteinuric gestational hypertension, chronic hypertension and exceptionally even in entirely normal pregnancies (Pijnenborg and Vercruysse et al., 2006). Perhaps variety of pregnancy-related pathology and heterogeneous genesis of PE could be explained by multiplicity of maternal health conditions (including genetic, life style and environment components), which interact with a certain extent of abnormalities in placentation. Possibly the minimal alteration in placentation may lead to PE in women with strong susceptibility and the other way around (Roberts and Gammill, 2005). That is why the current conception of the heterogeneous causes of PE is spread (Ness and Roberts, 1996).

Thus, the other component completing the development of PE is a maternal response to abnormal placentation. According to the two-stage theory, abnormal placentation must interact with maternal factors to result in the PE syndrome (Figure 1.1). Abnormal placentation leads to imbalance between constantly growing requirements of fetus/placenta/uterus and the limited abilities of placenta to give a necessary blood supply to fetus/placenta/uterus. Additionally, it was suggested that the ischemic placenta secrete soluble substances into maternal circulation, which are responsible for the symptomatic phase of PE, hallmarked by widespread maternal endothelial dysfunction. Among most well characterized factors in pathology of PE, the anti-angiogenic protein soluble fms-like tyrosine kinase-1 (sFlt-1), soluble endoglin (sEng), agonistic Ang II type-1 receptor autoantibodies (AT1-AA), inflammatory cytokines and oxidative stress, are of importance.

1.5.3.1 Angiogenic factors

Angiogenesis has a significant role in the placental development. Angiogenic factors are thought to be important in the pathogenesis of PE. Ligands and receptors of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) are vital in the regulation of angiogenesis (Keck et al., 1989; Maharaj et al., 2006) (Park et al., 1994).

Receptors of VEGF (VEGFRs), VEGFR-1 (Flk-1) and VEGFR-2 (KDR/Flk-1), predominantly involved in the regulation of blood vessels angiogenesis and expressed primarily on ECs. VEGFR-1 binds both VEGF and PlGF, whereas VEGFR-2 is specific to VEGF (Zachary, 2003). In pathogenesis of PE the growing interest focused on the soluble VEGF receptor-1 (sVEGFR-1, also known as sFlt-1). sFlt-1 is an endogenous protein produced by the placenta. sFlt-1 acts as a potent inhibitor of both VEGF and PlGF activity by binding them in the circulation and by this way preventing their interaction with endogenous receptors and as a result neutralizing their functions.

It has been suggested that an imbalance between pro-angiogenic and anti-angiogenic factors might be associated with the pathogenesis of PE. Indeed, the exaggerated level of sFlt-1 and low concentrations of free VEGF and PlGF are registered in serum of women with PE (Levine et al., 2004) (Shibata et al., 2005; Wikström et al., 2007). It has been confirmed that sFlt-1 level begin to rise about five to six weeks before the onset of clinical manifestation of PE (Maynard SE, 2003; Hertig et al., 2004) and it is

correlated with the severity of PE (Chaiworapongsa et al., 2004; Levine and Maynard et al., 2004). Moreover, recent data have shown association between alterations in angiogenesis and maternal endothelial dysfunction in women with PE (Sandrim et al., 2008).

The role of sFlt-1 in pathogenesis of PE is supported by animal studies. Administration of sFlt-1 to pregnant rats induced all clinical symptoms of PE - hypertension, proteinuria and glomerular endotheliosis (Maynard et al., 2003). The increased level of sFlt-1 has been found also in reduced uterine perfusion pressure (RUPP) model of PE (Gilbert et al., 2007; Makris et al., 2007).

The role of another circulating factor, sEng, in pathogenesis of PE has been suggested recently (Venkatesha et al., 2006). sEng is a co-receptor for transforming growth factor β (TGF- β), which prevents TGF- β binding to cell surface receptors and decreases endothelial NO signaling. sEng is produced by the ischemic placenta and significantly elevated in PE with a pattern similar to that for sFlt-1. The circulating sEng level is strongly correlated with severity of clinical signs of PE and its level was markedly increased already 2-3 months before the onset of PE (Levine et al., 2006).

There is growing evidence that VEGF, PlGF, and TGF- β are required for ECs maintenance in several tissues, including kidney and placenta. Enhanced placental secretion of sFlt1 and sEng inhibits VEGF and TGF- β 1 signalling in the vasculature of women with PE. This results in endothelial dysfunction. It should be noted that combined interaction of sFlt-1, PlGF, and sEng characterizes pathogenesis of PE better than any of them separately (Lim et al., 2008). Since the importance of VEGF, PlGF, sFlt-1, and sEng in pathogenesis of PE has been confirmed, these proteins could be targeted as a screening tool for prediction of PE and eventually for pro-angiogenic and anti-angiogenic therapies (Tjoa et al., 2007).

1.5.3.2 Angiotensin II type I receptor agonistic autoantibody

Ang II plays multiply roles in the pathology of PE, including contribution to hypertension, increased oxidative stress via production of superoxide anions, and activation of platelets (Shah, 2005). Several maternal symptoms of PE, i.e. hypertension and renal damage, could be attributed to an enhanced activation of Ang II receptor type 1 (AT1). However, it was a puzzling feature that the circulating Ang II levels were decreased in women with PE compared to normotensive pregnant women (Gant and Daley et al., 1973; Gant et al., 1980) (Shah, 2006). This contradiction was explained after discovery of the autoimmune antibody against the second extracellular loop (165–191) of AT1 receptor (AT1-AA) (Wallukat et al., 1999).

A number of recent studies have demonstrated elevated concentrations of AT1-AA in serum from women with PE in comparison with normal pregnancies (Xia et al., 2007; LaMarca et al., 2008; LaMarca et al., 2009). The AT1-AA was also detected in women with a history of PE up to 18 months after delivery (Hubel et al., 2007). Moreover, injection of immunoglobulin G (IgG) or purified the AT1-AA from PE women into pregnant mice induced hypertension and kidney damage (Zhou et al., 2008). Both placental ischemia and elevations of tumor necrosis factor- α (TNF- α) are considered as main stimuli for increased production of the AT1-AA (LaMarca et al., 2011). In experimental models of PE (the infusion of TNF α to RUPP rats) the levels of AT1-AA in the circulation was comparable with those in women with PE (LaMarca and Wallukat et al., 2008).

It appeared that AT1-AA has agonist-like effects and may be a reason for excessive AT1 receptor activation and could serve as a mediator of vascular injury in hypertension and PE (Yang et al., 2008). AT1-AA may cause hypertension by direct activation of AT1 receptors, whereas in PE additional pathways involving ET-1 and anti-angiogenic factors may also be implicated (LaMarca and Parrish et al., 2009). Indeed, a close association between enhanced production of AT1-AA, sFlt-1, sEng and placental oxidative stress has been demonstrated in animal studies (Zhou et al., 2008; Parrish et al., 2010; Parrish et al., 2011), although clinical data are fractional and controversial. Stepan et al. have failed to find the correlation between AT1-AA and sFlt-1 concentrations in women with PE (Stepan et al., 2006). However, more recent study has confirmed a strong positive correlation between serum concentration of AT1-AAs and sFlt-1 levels in women with severe PE (Siddiqui et al., 2010).

Current hypothesis is that chronic immune activation in association with placental ischemia leads to sFlt-1 and sEng overexpression via stimulation of the AT1-receptor possibly by production of the AT1-AA (LaMarca and Wallace et al., 2011). Thus, the potential role of AT1-AA in the development of hypertension in women with PE is a complex interaction between inflammatory, angiogenic and endothelial mediators produced excessively in response to placental ischemia.

1.5.3.3 Oxidative stress, inflammatory and metabolic responses

PE is associated with enhanced oxidative stress and exaggerated systemic inflammatory and metabolic responses. These states are closely interrelated. The activation of leukocytes in the circulation and increased their adhesion to ECs increases oxidative stress in PE, and vice versa, reactive oxygen species from other sources can initiate the inflammatory response. Indeed, hypoxia-induced transcription factor NF-kappaB and oxidative stress control inflammatory response (Li and Karin, 1999). Moreover, inflammation can trigger the metabolic changes, including insulin resistance and dyslipidemia with following increase of oxidative stress.

Increased oxidative stress due to the imbalance between lipid peroxidation and antioxidant defense mechanisms is a feature of PE. Indeed, maternal circulating and placental tissue levels of reactive oxygen species, and production rate of lipid peroxides are increased whereas several antioxidants are markedly decreased in women with PE (Siddiqui et al., 2010).

Oxidative stress in the placenta results in widespread placental lipid and protein oxidative modifications, mitochondrial and endoplasmic reticulum stress, tissue apoptosis and necrosis (James et al., 2010). Thus, oxidative stress further increases the damage of placenta and plays an important role in reduced placental perfusion in PE. However, the oxidative stress in PE is not localized to the placenta but disseminated into the maternal circulation and is a part of the systemic inflammatory response. Therefore, oxidative stress was postulated as the link between the early and late stages of the pathophysiology of PE (Roberts and Hubel, 1999). While the evidence provides a strong rationale for the therapy with antioxidants in PE, relatively recent trials of vitamins C and E in a number of different settings have not been successful (Poston L, 2006; Roberts et al., 2010; Xu et al., 2010).

Despite the immune system has a potential role in spiral artery remodelling in the first trimester, the systemic inflammatory stress plays also an important role in the

pathogenesis of PE in the third trimester (James and Whitley et al., 2010). Indeed, poor placentation increases the level of trophoblast debris into the maternal circulation, which elicits an increase inflammatory response and leads to endothelial dysfunction (Sargent et al., 2003). In PE both monocytes and granulocytes are activated with following increased adhesion to ECs and released of pro-inflammatory cytokines into the circulation (Janes and Goodall, 1994; Conrad et al., 1998; Sargent et al., 2006). It has been reported the higher circulating levels of TNF- α , IL-6, and IL-8 but the decreased IL-10 levels in PE compared to healthy pregnancy (Vince et al., 1995; Ellis et al., 2001) (Hennessy et al., 1999; Sharma et al., 2007) (Fabiana et al., 2008).

Finally, PE has many characteristic in common with the metabolic syndrome. The metabolic syndrome of PE includes hypertension, insulin resistance, complex changes in lipid profile, and inflammation (Sattar N, 1997; Hubel et al., 1998; Belo et al., 2002; Llurba et al., 2005) (Newstead et al., 2007). The same level of metabolic alterations are peculiar for atherosclerosis and reveal high risk for coronary artery disease (Carmenta et al., 2004). Since, elevated fasting insulin and glucose levels as well as proatherogenic lipid profile occur even before clinical manifestation of PE (Endresen et al., 1994) (Malek-Khosravi and Kaboudi, 2004), metabolic-mediated endothelial dysfunction has been considered as one of the major pathogenic pathway of PE for many years.

1.5.4 Maternal vascular dysfunction in preeclampsia

Several vascular changes have been reported in PE. Increased sympathetic tone has been shown in women with PE in comparison with normotensive pregnant and hypertensive non-pregnant women (Schobel et al., 1996; Stennett and Khalil, 2006). A higher constriction in response to NE (Nisell and Hjerdahl et al., 1985) and enhanced sensitivity to Ang II (Brown et al., 1997) were demonstrated in arteries of women with PE.

As soon as endothelial role in the control of arterial tone has been appreciated the investigations of vascular abnormalities during PE have been focused on endothelial dysfunction. Currently, endothelial dysfunction is considered as end-point of pathophysiological processes of PE and a key event in cardiovascular complications. Indeed, the primary clinical manifestations of PE such as hypertension, renal dysfunction and proteinuria, and edema in one way or another can be linked to alterations of endothelial function. The role of placental factors in this pathology seems to be significant. The factors produced by a poor oxygenated placenta may cause endothelial dysfunction due to imbalance between release of endothelium-derived relaxing and constricting factors with predominance in secretion of substances with vasoconstrictive properties (i.e., ET-1 and TX A₂) (Clark et al., 1992; Mills et al., 1999).

Endothelial dysfunction is accompanied by activation of coagulation cascade, increased vasoconstriction and intravascular fluid redistribution. This leads to reduce organ perfusion in the uterus (Roberts and Gammill, 2005) with secondary reduction of blood flow to the placenta/fetal unit (Pijnenborg and Vercruysse et al., 2006) creating a forward feedback cycle loop.

Several evidences of endothelial dysfunction in PE have been accumulated in the literature for the last decades. First of all, the presence of factors released by the injured or activated endothelium in the circulation of women with PE is well established. Indeed, increased circulating levels of von Willebrand factor (vWF, an

activator of platelet adhesion), tissue plasminogen activator (tPA, a fibrinolysis inhibitor), plasminogen activator inhibitor-1 (PAI-1), ET-1, cellular fibronectin, soluble vascular cell adhesion molecule, thrombomodulin and increased growth factor activity are reported in women with PE and altogether can be considered as remarkable evidences of endothelial disturbances or activation (Roberts et al., 1989; Friedman et al., 1995; Hayman et al., 1999). Some markers of endothelial dysfunction and activation have been noticed even before the clinical features of the PE.

Endothelial dysfunction in PE is confirmed by several functional studies on arteries from different vascular beds; although the heterogeneity in responses between studies should be noticed here as well. In general, reduced endothelium-dependent relaxation to ACh, BK and flow was shown in diverse small arteries *in vitro*. Namely, attenuated endothelium-dependent responses to BK, ACh and shear stress were reported in subcutaneous arteries from women with PE (Cockell and Poston, 1997) (Knock and Poston, 1996) (McCarthy et al., 1993). In contrast, similar responses to ACh and BK between normal pregnancy and PE or abolished ACh-, but preserved substance P-induced dilatations have been reported in subcutaneous arteries as well (VanWijk et al., 2002; Wimalasundera et al., 2005).

Also endothelial dysfunction was reported in omental arteries from women with PE (Pascoal et al., 1998; Suzuki et al., 2000; Suzuki et al., 2002). However, there was inconsistency between the studies and reduced endothelium-dependent responses to ACh but preserved to BK in one study (Pascoal and Lindheimer et al., 1998) contrasting with reduced responses to BK in another one (Suzuki and Kajikuri et al., 2000).

The controversial results were obtained on myometrial arteries as well. Using a wire myography technique a significant reduction of endothelium-dependent relaxation to BK has been shown in PE (Ashworth et al., 1997; Wareing et al., 2004; Wareing et al., 2006). However, after utilization of the pressure myography technique, Kenny et al. (2002) failed to reproduce the results (Kenny et al., 2002). In contrast, reduced response to BK (Svedas et al., 2002) and the lack of flow-induced dilatation (Kublickiene et al., 2000) were found in our laboratory. Thus, the discrepancy in results obtained in small arteries from different vascular beds in PE and between different research groups deserves consideration and requires further investigation.

Moreover, in contrast to above mentioned *in vitro* studies, the measurements of microcirculatory response in skin by laser Doppler perfusion monitoring and iontophoresis of ACh showed better endothelium-dependent response in women with PE compared with healthy pregnant women (Eneroth-Grimfors E, 1993; Davis et al., 2001).

Whereas endothelial dysfunction associated with PE at level of small arteries is generally appreciated, currently the main discussion is about possible mechanisms involved. Studies showing that serum from women with PE induce endothelial injury *in vitro* support the theory that circulating factors cause the endothelial dysfunction (Myers et al., 2005). Although abnormalities in every endothelium-derived factor are reported in PE (Bird et al., 2003; Gillham et al., 2003; Poniedziałek-Czajkowska et al., 2011), there are not enough evidences so far to make a clear statement about predominant contribution of NO, PGI₂, and EDHF into compromised endothelium-dependent relaxation of small human arteries in PE.

1.6 LONG TERM CONSEQUENCES OF PREECLAMPSIA

The hypothesis that women with a history of PE are more prone to CVD later in life has been originated long time ago. As early as 50 years ago the first investigation of previously PE women was performed in order to estimate the effect of PE on blood pressure later in life (Adams and MacGillivray, 1961). For the following half a century a number of studies have been carried out worldwide to investigate the relationship between PE and long-term cardiovascular consequences such as hypertension, ischemic heart disease, stroke, and venous thromboembolism, diabetes Mellitus, renal disease as well as cancer.

Recently, Bellamy *et al* conducted meta-analysis where overall 25 prospective and retrospective cohort studies were included (Bellamy et al., 2007). Those studies numbered 29 459 incident cases of CVD from 198 252 cases of PE. The control group included over 3 million women. It was summarized that a history of PE enhances risk of CVD in follow-up of 10-14 years (Bellamy and Casas et al., 2007). The relative risk (RR, 95% confidence intervals (CI)) for hypertension was 3.70 (2.70 to 5.05); for ischemic heart disease 2.16 (1.86 to 2.52), for stroke 1.81 (1.45 to 2.27) and for venous thromboembolism 1.79 (1.37 to 2.33) compared with women who have had healthy pregnancies. These findings have not been adjusted for the severity and recurrence of PE and/or fetal outcome. In parallel with estimation of long-term effects of PE on CVD, no association between PE and future breast cancer has been found.

More recent meta-analyses has included 5 case-control studies and 10 cohort studies, involving a total of 118 990 women with a history of PE/eclampsia and 2 259 576 women with a history of uncomplicated pregnancies (McDonald et al., 2008). The risk of CVD appeared to relate to PE severity. Thus, the RR (95% CI) was 2.00 (1.83-2.19) for mild PE, 2.99 (2.51-3.58) for moderate PE and 5.36 (3.96-7.27) for severe PE (complicated by preterm delivery and/or fetal death) (McDonald and Malinowski et al., 2008).

Analyzing more than one million delivers, Lykke et al. have shown strong association between PE and subsequent hypertension. Besides of the severity, parity and recurrence of PE increased the risk of subsequent hypertension. Thus, women having 2 pregnancies both complicated by PE had a 6.00-fold (5.40 to 6.67) increased risk of subsequent hypertension compared with 2.70-fold (2.51 to 2.90) for women having PE in their first pregnancy only and 4.34-fold (3.98 to 4.74) for women having PE in their second pregnancy only (hazard ratios are presented with 95% CI) (Lykke et al., 2009).

Previous studies have shown that women within 3-7 years after PE have an increase incidence of microalbuminuria, a potential marker of both CVD and renal damage (Nisell H, 1995; Bar et al., 1999). The recent epidemiological Norwegian study has shown that women with previous PE in their fist pregnancy were 4.7 times (3.6-6.1; 95% CI) more likely to develop ESRD later in life then those with uncomplicated pregnancies. The even greater risk was found in those women who had PE in more than one pregnancy or in case when PE was complicated by IUGR (Vikse et al., 2008).

In spite that the link of PE and later CVD complications is obvious, relationships between causality and consequence are still under discussion. So far we cannot answer the question whether residual “damage” from PE induces later life diseases or PE unmasks predisposition of women to these diseases, since we have too little information available on women health before their PE pregnancy. Nowadays we have only indirect evidences, which allow assuming that some women have susceptibility to develop PE as well as CVD in later life. Increased pre-pregnancy serum levels of triglycerides, cholesterol, LDL cholesterol, non-high density lipoprotein cholesterol, and blood pressure were associated with an increased risk for developing of complications in their pregnancies. The odds ratio (95% CI) for developing of PE in women with baseline systolic blood pressures greater than 130 mm Hg was 7.3 (3.1 to 17.2) compared with normotensive women independently from the parity (Magnussen et al., 2007). This suggests that women with prevalence of cardiovascular risk factors may manifest clinical symptoms of PE in their pregnancy.

Hereby at this stage, the pregnancy should be considered as a screening test for later life CVD/ESRD. Therefore, health of women with a history of PE has to be supervised regularly later on, since they failed ones the pregnancy test and may be considered as a risk group.

1.6.1 Endothelial function in follow-up preeclampsia

Even if PE appears to begin in the placenta, the target maternal organ is endothelium. The epidemiological evidences of the link between PE and increased prevalence of CVD later on has drawn an interest to investigations of vascular function in women experienced PE. The high level of cardiovascular abnormalities among these women could be referring to a generalized maternal endothelial dysfunction. Few studies have been performed to determine if endothelial dysfunction persists after the PE pregnancy. The data of *in vivo* studies are summarized in Table 1. The postpartum period from months to 25 years has been covered and different technical approaches have been used. It should be noted that heterogeneity of PE groups (combination of early or late onset of PE in the same group) is a feature of majority of studies. As a consequence, the available results are contradictory.

Noninvasive measurement of arterial elasticity might be considered as an indirect method for evaluation of endothelial function as well. It is well accepted that reduced arterial elasticity is associated with endothelial dysfunction. An increase in arterial stiffness has been documented in high vascular risk conditions such as hypertension (McVeigh et al., 1991), diabetes mellitus (Dogra et al., 2006) and CKD (Wilson et al., 2004). Furthermore, arterial stiffness has been shown to be strongly associated with an increased cardiovascular risk (Boutouyrie et al., 2002) (Laurent S, 2007; Vlachopoulos et al., 2010).

Studies in women with a history of PE have reported conflicting results indicating either no difference in arterial compliance (Rönnback et al., 2005; Spasojevic et al., 2005; Lampinen KH, 2006) or an increase of arterial stiffness (Elvan-Taspinar A; Pàez et al., 2009; Robb et al., 2009; Yinon et al., 2010; Souwer et al., 2011). Such inconsistency might be due to the fact, that these studies assessed different parameters of arterial stiffness without comprehensively adjusting for all known confounding variables such as blood pressure, heart rate, body mass index (BMI), postpartum time frames, heterogeneity of PE and etc.

Table 1. Summary of *in vivo* studies of vascular function after preeclampsia

Study	Study group	N Cases (Controls)	Time after delivery	Methods	Results				
					EDV	EIV	BP	BMI	Other
Macrocirculation									
Yinon et al., 2010	ePE, /PE	24 (16)	6-24 months	Ultrasound, brachial					= {glucose, insulin, HOMA index, TC, HDL-, LDL-cholesterol, TG, CRP, microalbumin/creatinine ratio, sFlt, sEng, VEGF, PlGF}; ↑arterial stiffness
				FMD, glyceryl trinitrate (GTN)	↓	=	↑	=	
					=	=	↑	=	
Hamad RR, 2007	ePE	18(17)	1-1.5 years	Ultrasound, brachial					= {VLDL-, LDL-, HDL-cholesterol, VLDL-, LDL-, HDL-triglyceride, proinsulin, insulin, glucose, IGF-1, IGFBP-1, IGFBP-3, free IGF-1, C-peptide, Lp(a), fibrinogen, PAI-1, tPA, vWF, hsCRP, ICAM-1, activated factor VII, VCAM-1, E-selectin}
				FMD, glyceryl trinitrate (GTN)	↓	↓	↑	↑	
Germain et al., 2007	sPE	25(22)	1-2 years	Ultrasound, brachial					↑ TC, ↓nitrites.
Paez et al., 2009	PE	20(20)	2-3 years	FMD, glyceryl trinitrate (GTN)	↓	=	↑	=	= {L-Argenin, ADMA, VEGF, sFlt-1}
				Ultrasound, brachial					= {glucose, insulin, TG, HDL-and LDL-cholesterol};
				FMD; glyceryl trinitrate (GTN)	↓	=	=	=	↑arterial stiffness
Chambers et al., 2001	PE, Recurrent PE	113 (48)	3 years	Ultrasound, brachial					↑ total to HDL chol ratio, ↑ E-selectin, ↑waist/hip ratio,
				FMD, glyceryl trinitrate (GTN)	↓	=	↑	↑	= {total, HDL cholesterol, TG, glucose, total homocystein, ICAM-1}

Study group	N	Cas- es (Con- trols)	Time after delivery	Methods	Results				
					EDV	EIV	BP	BMI	Other
Peripheral circulation									
Lommerse et al., 2007	PE	32(10)	6 months	VOP, reactive hyperemic forearm blood flow	=	NA	=	=	= {pulse pressure, LV mass, insulin, HDL chol, HOMA index, GFR glomerular filtration rate}
Agatsuma et al., 2004	PE	16 (14/20)	6-12 months	VOP, stress induced forearm blood flow	↓	NA	↑	↑	NA ↑total vascular resistance; = {cellular fibronectin, E-selectin, Apo-B, free fat acids, TC, HDL, glycerol, insulin, glucose, HOMA index, malondialdehyde}
Evans et al., 2011	PE	18(50)	16 months	VOP, stress induced forearm blood flow	↓	NA	↑	=	= left ventricular properties = arterial compliance
Lampinen et al., 2006	PE	30(21)	5-6 years	VOP, forearm blood flow - infusion of ACh and SNP	↓	↓	↑	=	= in arterial stiffness ↑ HOMA index;
Mangos et al., 2012	PE	39(35)	2-12 years	VOP, reactive hyperemic forearm blood flow	=	NA	↑	=	= {sympathetic activity, glucose, total, HDL-, LDL-cholesterol, TG, GFR, albumin /creatinine}

Study	Study group	N Cases (Controls)	Time after delivery	Methods	Results				
					EDV	EIV	BP	BMI	Other
Microcirculation									
Khan et al., 2005	PE	15(54)	6 weeks	Laser Doppler, intra-foresis of Ach and SNP	=	=	NA	NA	NA
Blaauw et al., 2005	ePE	25(23)	3-11 months	Laser Doppler, intra-foresis of Ach and SNP	↑	=	↑	↑	NA
Kvehaugen et al., 2011	PE, PE+SGA	26(15)	5-8 years	EndoPAT-2000 (reactive hyperemia)	= in PE; ↓ in PE+SGA	NA	↑	=	↑hsCRP,↑sFlit-1, =calprotectin, sEng, VEGF, PlGF, TG, TC, HDL, LDL}
Spaan et al., 2010	PE	22(29)	23 years	Laser Doppler flowmetry (reactive hyperemia)	=	NA	↑	=	↑ insulin, ↑HOMA-IR, = { HbA1c; CRP, total, HDL-, LDL-cholesterol, TG}
Ramsay et al., 2003	PE	10(10)	15-25 years	Laser Doppler, intra-foresis of Ach and SNP	↓	↓	↑	=	NA

↑ – increase; ↓ – decrease; = – no difference; NA – not applied.

PE – preeclampsia; EDV – endothelium-dependent vasodilatation; EIV – endothelium independent vasodilatation; BP – blood pressure; BMI – body mass index; ePE – early-onset PE, /PE – late-onset PE; sPE – severe PE; Ach – acetylcholine; SNP – sodium nitroprusside; GTN- glyceryl trinitrate; FMD – flow mediated dilatation; FBF – forearm blood flow.

TC – total cholesterol; TG – triglyceride; HDL – high-density lipoprotein; LDL – low-density lipoprotein; VLDL – very low-density lipoprotein; Apo-B – Apolipoprotein B; ApoA1 – Apolipoprotein A-1; hsCRP – highly sensitive C-reactive protein; ICAM-1 – intracellular adhesion molecule 1; IGF-I – insulin-like growth factor I; IGFBP – insulin-like growth factor binding protein; HbA1c – glycated hemoglobin; PAI-1 – plasminogen activator inhibitor 1; VCAM-1 – vascular cell adhesion molecule 1; vWF – Von Willebrand factor; GFR – glomerular filtration rate; ADMA – asymmetric dimethylarginine; sEng – soluble endoglin; VEGF – vascular endothelial growth factor; PlGF – placental growth factor; SFlit-1 – soluble fms-like tyrosine kinase; LV mass – Left Ventricular Mass; SGA – small for gestational age; HOMA – Homeostatic Model Assessment.

Thus, although until now a number of studies for direct or indirect evaluation of endothelial function in women with a history of PE were conducted, the inconsistency in the results and heterogeneity of recruited participants have limited the significance of the obtained data. Therefore, more investigations of vascular function in the well defined group of women with a history of PE adjusted for the postpartum period and the severity of PE are still needed. Moreover, there are no studies so far for investigation of vascular function on isolated arteries from women with a history of PE. Such type of investigation could help to distinguish the alterations within the vascular wall vs. systemic changes observed in these women and eventually understand the mechanisms of dysfunction restricted to the vascular wall performance.

2 AIMS

The general aim of the investigations summarized in this thesis was to extend the current knowledge about structural and functional properties of human resistance arteries with particular focus on endothelial regulation of vascular tone and the mechanisms of endothelium-dependent dilatation in health and disease.

The specific aims of the *ex vivo* studies were:

Study I:

- To investigate structural (wall thickness, wall/lumen ratio and CSA) and functional (dilatation, constriction and elastic properties) characteristics of subcutaneous resistance arteries from ESRD patients;
- To examine the role of NO in flow-induced responses in resistance arteries from ESRD patients vs. controls;
- To understand the potential mechanism/s of endothelial dysfunction in ESRD.

Studies II-III:

- To assess the endothelial function of subcutaneous and myometrial resistance arteries from women with preeclampsia;
- To compare the relative contribution of EDHF vs. NO into overall endothelium-dependent dilatation between arteries from women with vs. without preeclampsia;
- To clarify the specific mechanisms involved in EDHF-mediated relaxation in preeclampsia.

Study IV:

- To investigate structural (wall thickness, wall/lumen ratio and CSA) and functional (dilatation, constriction and elastic properties) characteristics of subcutaneous resistance arteries from women with a history of early-onset preeclampsia;
- To examine the role of NO in dilatation and constriction of resistance arteries from women with a history of early-onset preeclampsia vs. women with a history of normal pregnancy.

3 MATERIALS AND METHODS

Three main groups of participants were included in this thesis. Each group contained two subgroups: case (ESRD patients, Study I; pregnant women with PE, Studies II-III; volunteers with history of severe early-onset PE, Study IV) and matched controls. The Ethical Committee at Karolinska University Hospital, Stockholm approved the protocols for all studies. All participants gave writing informed consent prior to tissue biopsy and blood collection. Figure 3.1. summarizes the number of the subjects included in the studies, used material and applied methods.

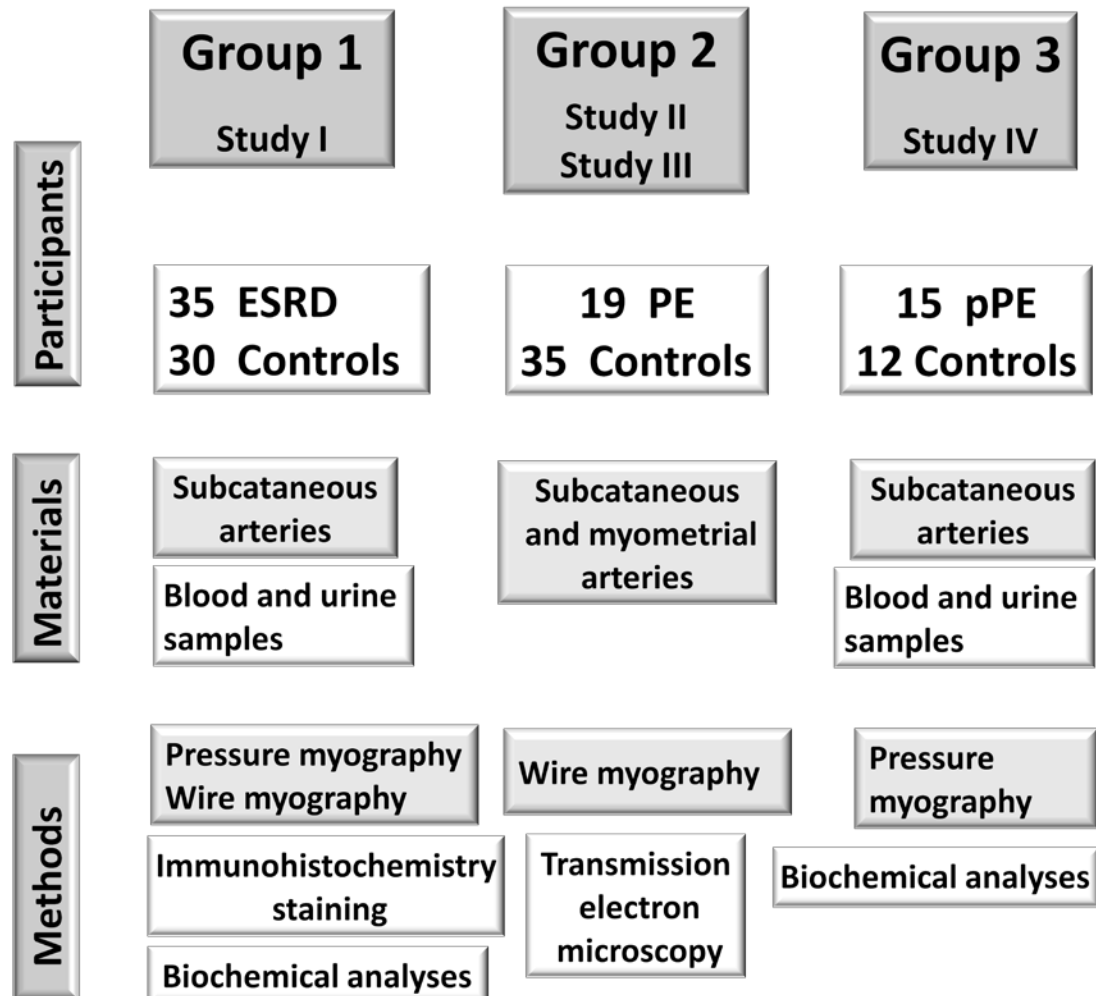


Figure 3.1. Study design: distribution of participants, materials and applied methods within the project. ESRD, end-stage renal disease; PE, preeclampsia; pPE, previous preeclampsia.

3.1 PARTICIPANTS

Study I. Thirty five ESRD patients (median age 58 years; 71% males) who underwent peritoneal dialysis catheter insertion were recruited at the Division of Renal Medicine at Karolinska University Hospital, Huddinge. In order to establish homogeneity, only patients starting dialysis treatment *de novo* were included. Thirty gender-matched volunteers (median age 54 years; 77% males) who underwent a surgery for hernia

repair or laparoscopic cholecystectomy were recruited as controls at the Division of Surgery at Karolinska University Hospital, Huddinge.

Clinical history of CVD or diabetes and medical treatments were obtained from medical records. CVD was defined as the presence of ischaemic cardiac disease, peripheral vascular disease and/or cerebrovascular disease. Blood pressure and smoking status were recorded.

Studies II-III. Nineteen women with PE pregnancies (8 women included in both studies plus 7 women for Study II and 4 for Study III) and 35 women with normal pregnancies (23 in Study II and 12 in Study III) were recruited from the Division of Obstetrics and Gynecology at Karolinska University Hospital, Huddinge. PE was defined according to the criteria of the International Society for the Study of Hypertension in Pregnancy. Diagnosis required blood pressure $\geq 140/90$ mmHg after the 20th week of gestation in association with >300 mg proteinuria in a 24-h urine collection. Two women with PE fulfilled the criteria for HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count). Biopsies were obtained during emergency cesarean delivery due to deterioration of PE. All women from the control group were delivered by elective cesarean section due to previous cesarean delivery ($n=10$), psychological reasons ($n=15$), and breach presentation ($n=10$). None had any history of diabetes mellitus, abnormal renal or hepatic function, established hypertension and atherosclerosis, malignancy, systemic infection, vasculitis, and recent surgery or trauma.

Study IV. Women with last singleton pregnancy complicated by severe early-onset (delivery at ≤ 34 weeks of gestation) PE, who had delivered between years 2007 and 2010 were included in the study. Severe PE was defined (based on the international classification of diseases (ICD-10) as blood pressure $\geq 160/110$ mmHg on 2 occasions at least 6 hours apart and/or proteinuria of more than 5 g in a 24-hour urine collection and/or organ damage (ICD-10). Twelve women experienced uncomplicated singleton pregnancies matched for age, BMI, smoking status and time after delivery comprised a control group. Exclusion criteria for both groups were: age ≥ 45 years, ongoing pregnancy, CVD, obesity, diabetes mellitus, chronic hypertension, malignancy, hepatic or renal disease, systemic infection, vasculitis, venous thromboembolic disease, recent surgery, trauma or presence of hormonal contraception. Majority of women included in the study were in the early follicular phase of the menstrual cycle.

The participant's information regarding to previous pregnancy and family history of preeclampsia and CVD were collected. Height and weight were recorded. Waist circumference was measured halfway between the rib cage and the pelvic bone. Hip circumference was measured at the maximal circumference of the hips. BMI, waist-to-hip ratio were calculated. Blood pressure was measured twice using a sphygmomanometer. The mean of these two measurements was used in the analyses.

3.2 MATERIALS

3.2.1 Blood samples

Blood samples were collected after an overnight fast. Plasma samples and serum were stored at -70°C pending further analyses.

Study I. Glomerular filtration rate was estimated by the mean of creatinine and urea clearances in ESRD, whereas cystatin-C was used to estimate glomerular filtration rate in controls. Serum IL-6 was measured on an Immulite® analyser (Siemens Medical Solution Diagnostic). Circulating ADMA was assessed in serum using commercial ELISA assays (DLD Diagnostika). Serum concentrations of albumin (by the Bromocresol Purple method), creatinine, haemoglobin, blood lipids and high sensitivity C-reactive protein (hsCRP) were measured by routine procedures at the Department of Clinical Chemistry at Karolinska University Hospital-Huddinge.

Study IV. Biochemical analyses of blood included estimation of lipid profile, glucose homeostasis, inflammation, homocysteine. Urine albumin, creatinine and albumin/creatinine ration were detected.

3.2.2 Artery preparation

Subcutaneous fat or myometrial biopsies ($\approx 2 \times 1.5 \times 1.5$ cm), in accordance with study design, were obtained from the participants. Removed biopsy was immediately placed into an ice physiological salt solution (PSS). Small subcutaneous and/or myometrial arteries ($\approx 200 \mu\text{m}$) were identified and dissected from the biopsies by carefully removing surrounding tissue using a stereomicroscope. The dissected arteries were divided into segments with a length of approximately $2 \mu\text{m}$ for mounting in the wire-myography system and approximately the same length but with particular control for the lack of branches for mounting in the pressure myography systems.

3.3 METHODS

3.3.1 Pressure myography

The arterial segments were oriented to mimic the direction of flow *in vivo* and mounted between two glass microcannulae in a pressure myograph chamber (Living Systems Instrumentation Inc., USA; Figure 3.2.a). Intraluminal pressure (60 mmHg) was maintained by a servo-controlled pump. The constant pressure in the system confirms the absent of arterial leakage, otherwise the leaked arteries were discarded. The dimensions of the cannulated artery (internal diameter and wall thickness) were continuously monitored via a video dimension analyser. The organ bath was superfused with $\approx 37^\circ\text{C}$ PSS gassed with 5% CO_2 in O_2 . Each artery was equilibrated for 60 min. The viability tests were carried out by examining the responses to NE ($1 \mu\text{mol/l}$), and endothelial function was confirmed by relaxation to ACh ($1 \mu\text{mol/l}$).

3.3.2 Wire myography

Small subcutaneous and myometrial arteries were mounted on two stainless steel wires (25–40 μm in diameter) in the organ baths of a four-channel wire myograph (multimyograph, model no. 610; Danish Myo Technology; Aarhus, Denmark; Figure 3.2.b) as described previously (Luksha et al., 2004). Each organ bath contained warmed (37°C) PSS and was continuously bubbled with 5% CO_2 in O_2 . Following a 30-min equilibration period, a passive circumference-tension curve was created for each segment to set optimum resting tension. This resting tension is calculated to simulate an *in vivo* transmural pressure of 100 mmHg. Arteries were then set at 90% of this tension to enable optimal contractile conditions with a low resting tension. Calibration and data processing were performed using Myodac software (version 2.1, Danish Myo Technology). All solutions were refreshed every 30 min. Five reference constrictions

were elicited. The first, second, and fifth contractions were produced using a high (124 mmol/l) potassium physiological salt solution (KPSS, made by equimolar substitution of KCl for NaCl in PSS) containing NE (1 μ mol/l). The third was obtained with NE (1 μ mol/l) alone and the fourth with KPSS alone. Arteries that failed to produce active tension equivalent to 100 mmHg when constricted with KPSS containing 1 μ mol/l NE were discarded. Arteries that did not fulfill the viability criteria (i.e. >60% relaxation to ACh (1 μ mol/l) after pre-constriction with NE (1 μ mol/l)) were discarded from experiment.

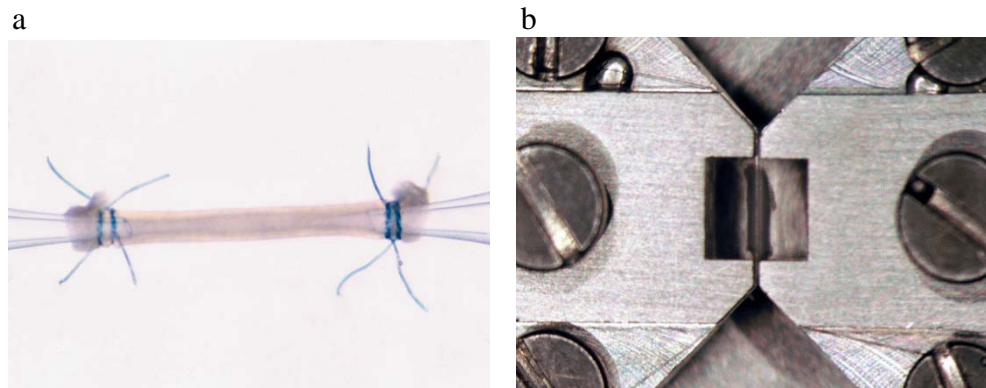


Figure 3.2. Arteries mounted in the pressure (a) and wire (b) myographies.

3.3.3 Experimental protocols

3.3.3.1 Assessment of vascular function: flow-mediated dilatation

After the equilibration period, the intraluminal pressure was gradually increased from 60 to 80 mmHg for arteries with internal diameter >200 μ m, and the internal diameter was recorded after 20 min. In contrast, for arteries with an internal diameter <200 μ m, intraluminal pressure was permanently kept at 60 mmHg. A flow response curve to stepwise increase of intraluminal flow every 3 min from 0 to 180 μ l/min in Study I and from 0 to 90 μ l/min in Study IV was performed on the pre-constricted artery to \approx 50% of the initial diameter. In a separate experimental setup in Study I and in all experiments in Study IV flow response curves were obtained before and after incubation with NO synthase (NOS) inhibitor, *N*^o-nitro-L-arginine-methyl ester (L-NAME) (300 μ mol/l, 30 min).

3.3.3.2 Assessment of agonist-induced responses on the pressure myography

Equilibrated arteries at 60 mmHg were superfused with NE (1 μ mol/l) for 20 min in order to induce a stable constriction. Concentration-response curves were obtained using incremental concentrations of vasodilators ACh (endothelium-dependent agonist, 3 nmol/l–1 μ mol/l) and sodium nitroprusside (SNP, endothelium-independent agonist, 0.1 μ mol/l–0.1 mmol/l) in Study I and vasodilators BK (endothelium-dependent agonist, 1 nmol/l–3 μ mol/l) and SNP (0.1 μ mol/l–0.1 mmol/l) in Study IV. ACh, BK or SNP were added in NE-contained PSS. The concentration-response curves using incremental concentrations of vasoconstrictors NE (non-selective agonist of adrenergic receptors, 1 nmol/l–1 μ mol/l), Ang II (0.01nmol/l–30 nmol/l) were assessed in Study IV. The concentration-response curves to NE, BK, Ang II were repeated after incubation with the NOS inhibitor L-NAME (300 μ mol/l, 30 min) in Study IV. Each

concentration of the agonist was extraluminally perfused for 3 min, while the changes in the diameter were constantly recorded.

3.3.3.3 Assessment of pressure-induced myogenic response and the basal tone

After 20 min artery equilibration at 20 mmHg, intraluminal pressure was gradually increased up to 120 mmHg. Internal artery diameter was recorded after each pressure increment, which was maintained for 5–7 min in order to reach a steady state diameter. Thereafter, PSS was replaced with PSS without Ca^{2+} (Ca^{2+} -free PSS) to determine the passive diameter curve in response to stepwise pressure increase.

To estimate the basal vascular tone at resting condition (at 60 mmHg) internal artery diameter was recorded after equilibrated period (60 min) in PSS. As well the passive arterial diameter at 60 mmHg was recorded in Ca^{2+} -free PSS.

3.3.3.4 Assessment of arterial distensibility

PSS was replaced for Ca^{2+} free PSS. Intraluminal pressure was decreased to 5 mmHg and after 20 min of equilibration the internal diameter was recorded. Thereafter the intraluminal pressure was increased stepwise (10 mmHg pressure increment) up to 120 mmHg in the Study I and up to 160 mmHg in Study IV. The internal diameter of each pressure step was recorded after 2 min.

3.3.3.5 Assessment of agonist-induced constriction on the wire myography in Study I

The cumulative concentration-response curves were constructed for phenylephrine (selective agonist of α_1 -adrenergic receptors, 10 nmol/l–0.03 mmol/l), NE (10 nmol/l–0.03 mmol/l), Ang II (0.1–30 nmol/l) or ET-1 (0.1–30 nmol/l) before and after incubation with the NOS inhibitor L-NAME (300 $\mu\text{mol/l}$, 30 min).

3.3.3.6 Assessment of BK-induced response and contribution of EDHF to BK-mediated relaxation in Studies II-III

All arteries were pre-constricted with NE (3 $\mu\text{mol/l}$) or vasopressin (VP; 3 nmol/l) and the concentration-response curves were obtained using incremental concentrations of BK (1 nmol/l–3 $\mu\text{mol/l}$). Arteries were then incubated for 20 min with L-NAME (300 $\mu\text{mol/l}$) and the COX inhibitor indomethacin (Indo; 10 $\mu\text{mol/l}$) to block the production of NO and PGI_2 , respectively. Subsequently, arteries were pre-constricted again and concentration-response curve for BK was repeated. The term “EDHF” used in this study refers to the L-NAME- and Indo-insensitive component of endothelium-dependent vasodilatation in response to BK.

Assessment of contribution of AA metabolites, H_2O_2 , and MEGJ to EDHF-type responses

To investigate whether AA metabolites played a role as EDHF, the BK-induced relaxation was assessed in the presence of the specific inhibitor of CYP450 epoxigenase (CYP2C9) sulfaphenazole (10 $\mu\text{mol/l}$, 30 min) in combination with L-NAME + Indo.

To evaluate if H_2O_2 was involved in the EDHF-typed response, the BK-induced relaxation was assessed in the presence of catalase (1250-6250 U/ml, an enzyme, which degrades H_2O_2 forming H_2O and O_2) in combination with L-NAME + Indo.

To elucidate the contribution of gap junctions to the EDHF-typed response, the concentration response curves to BK were obtained after incubation with a reversible inhibitor of gap junctions - 18- α -glycyrrhetic acid (18- α GA, 100 $\mu\text{mol/l}$, 15 min) in combination with L-NAME + Indo.

3.3.4 Immunohistochemistry

Immunohistochemistry (IHC) was used to determine the level of HSPs 90, 70, 27 and the oxidative stress marker nitrotyrosine in isolated small subcutaneous arteries from patients with ESRD and compared them with controls. The control group was composed from the patients included or not included in the functional study. Before the experiments, transverse 7 μm cryosections were cut using a cryostat HM 500 M, mounted onto Superfrost slides, and stored at -20°C . The experiments were carried on in two-day cycles. During the first day the samples were thawed for 5 to 10 min, fixed with pre-cooled formaldehyde for 4-5 min. Blocking was carried out with 0.75% H_2O_2 in phosphate buffered saline (PBS) and 4% normal goat serum (NGS) in PBS, each for 30 min incubation time at room temperature. Then the primary antibodies, diluted in PBS with 0.1% Tween and 3% NGS were added on the sections and the slides were incubated overnight at $+4^\circ\text{C}$. Antibody concentration was 10 $\mu\text{g/ml}$ for anti-HSP90, 0.66 $\mu\text{g/ml}$ for anti-HSP70 and 20 $\mu\text{g/ml}$ for anti- nitrotyrosine. Negative controls with 0.1% Tween in 3% PBS and in PBS without primary antibodies were used. During the second day, the secondary antibody (5 $\mu\text{g/ml}$) diluted in PBS with 3% NGS was added to the sections. The slides were incubated for 30 min at room temperature. Streptavidin-Biotin Peroxidase solution on 30 min incubation and 3,3'-Diaminobenzidine (DAB) solution on 2 min incubation were successively added. Between each step the slides were washed with PBS, and after the DAB incubation they were accurately washed in dH_2O . After nuclear staining with 10% hematoxylin for 2 min the sections were washed with hot running water and dehydrated with increasing concentrations of ethanol (70% x 5 min, 95% x 5 min and 99.5% x 10 min), immersed in xylene for 15 min and then mounted with Pertex mounting medium.

3.3.5 Transmission electron microscopy

Artery segments were fixed in 2% glutaraldehyde + 0.5% paraformaldehyde in 0.1 M sodium cacodylate buffer containing 0.1 M sucrose and 3 mM CaCl_2 , pH 7.4, at room temperature for 30 min followed by 24 h at 4°C . Specimens were rinsed in 0.15 M sodium cacodylate buffer containing 3 mM CaCl_2 , pH 7.4; postfixed in 2% osmium tetroxide in 0.07 M sodium cacodylate buffer containing 1.5 mM CaCl_2 , pH 7.4, at 4°C for 2 h; and dehydrated in ethanol followed by acetone and embedded in LX-112 (Ladd, Burlington, VT). Semi-thin sections were cut and stained with toluidin blue and used for light microscopic analysis. Sections were examined in a Tecnai 10 transmission electron microscope at 80 kV, and digital images were captured by a Mega View III digital camera (Soft Imaging System, Muenster, Germany).

3.4 CHEMICALS AND SOLUTIONS

The composition of PSS (mmol/l) was NaCl 119, KCl 4.7, CaCl_2 2.5, MgSO_4 1.17, NaHCO_3 25, KH_2PO_4 1.18, ethylenediamine-tetraacetic acid 0.026, and glucose 5.5;

pH 7.4. Relaxing solution was Ca^{2+} -free PSS supplemented with papaverine (0.1 mmol/l) and ethylene glycol-bis-(β -aminoethyl ether) tetraacetic acid (1 mmol/l). NE was dissolved directly in PSS, whereas for ACh, phenylephrine, Ang II, ET-1, SNP and sulfaphenazole stock solutions were prepared in distilled water and further dissolved in PSS. Indo, ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, selective guanylate cyclase inhibitor), pinacidil were dissolved in ethanol, and 18- α GA was dissolved in dimethyl sulfoxide. All chemicals were obtained from Sigma-Aldrich Sweden AB (Sweden). Monoclonal mouse antibodies used for HSP27 (ab8600), HSP70 (ab6535), HSP90 (ab1429) and nitrotyrosine (ab7048), and secondary biotinylated polyclonal goat anti-mouse IgG antibody (ab6788) were obtained from Abcam plc (UK, Cambridge).

3.5 CALCULATIONS

Pressure myography: Constriction to NE, AngII was calculated as a percentage change from initial diameter. Relaxation to flow, ACh, BK and SNP were calculated as a percentage change in internal diameter in response to stimulation, divided by the difference in internal diameter before and after pre-constriction.

Wall shear stress (τ , dyn/cm²) was calculated using the Hagen-Poiseuille formula:

$$\tau = 4 \times \eta \times Q \times 10^9 / \pi r^3,$$

where η is viscosity of the perfusate (poise – dyn s/cm²), Q is flow rate (μ l/s) and r is artery radius (μ m). The factor of 10^9 in the equation is to correct the use of μ l/s for flow and μ m for artery radius ($1 \mu\text{l} = 10^9 \mu\text{m}^3$). Viscosity of PSS was assumed as 0.007 poise at 37°C.

Cross-sectional area (CSA) was calculated as:

$$CSA = (\pi/4) \times (D_e^2 - D_i^2),$$

where D_e and D_i are external and internal diameters, respectively.

Wall/lumen ratio was defined as:

$$\text{Wall thickness} / D_i.$$

The tone developed by artery at resting condition (60 mmHg, basal tone) and pressure-induced myogenic tone was calculated as:

$$\text{Basal tone (\%)} = 100 \times (D_{60 \text{ Ca}^{2+}\text{-free PSS}} - D_{60 \text{ PSS}}) / D_{60 \text{ Ca}^{2+}\text{-free PSS}},$$

$$\text{Myogenic tone (\%)} = 100 \times (D_{i \text{ Ca}^{2+}\text{-free PSS}} - D_{i \text{ PSS}}) / D_{i \text{ Ca}^{2+}\text{-free PSS}},$$

where D_i is the internal diameter of the artery, respectively.

Distensibility index was calculated as:

$$\text{Distensibility index (\%)} = (D_i D_0) \times 100,$$

where D_i is the internal diameter in Ca^{2+} -free PSS at different steps of pressure and D_0 is the internal diameter in Ca^{2+} -free PSS at 5 mmHg.

Wire myography: The force developed by the artery per square mm of artery wall during application of a certain concentration of a vasoactive substance was calculated using Myodata (version 2.1, Danish Myo Technology). Constriction response was expressed as a percentage of the maximal constriction with KPSS. The relaxation to agonist was calculated as a percentage of the constriction. Negative log concentration required to achieve 50% of the maximum response (pEC_{50}) was calculated by nonlinear regression analysis (BioDataFit 1.02).

The homeostatic model assessment (HOMA) index was calculated as fasting insulin concentration ($\mu\text{U/mL}$) \times fasting glucose concentration (mmol/L)/22.5.

3.6 STATISTICAL ANALYSIS

Baseline characteristics of the patients and arteries used were analyzed by conventional parametric and non-parametric methods where appropriate. Analysis of variance (ANOVA) for repeated measures was used to compare response to stimulation before and after incubation with inhibitors and for differences in stimulus responses between the experimental groups. Student's *t*-test was used to compare pEC_{50} value. Spearman's rank correlation was used to evaluate univariate correlation.

The semi-quantitative analysis for ICH staining intensity was assessed by the average blind score of 3 observers: using a scale between 0 and 3, where 0 was absence of staining and 3 corresponded to maximal intensity. In addition, a computer image analysis software (ImageJ), available at <http://rsb.info.nih.gov/ij/index.html>, was used for quantitative analysis of immunostaining (Rangan and Tesch, 2007; Takacs et al., 2008). The results were evaluated with Mann-Whitney U test for non-parametric comparisons. Statistical analyses were performed using STATISTICA (version 7.0 and 8.0, StatSoft, Uppsala, Sweden). All comparisons were considered statistically significant if $P < 0.05$.

4 RESULTS AND DISCUSSION

4.1 STUDY I (ESRD)

4.1.1 Clinical data

Age, gender and smoking status were similar between the groups (Table 1, Paper I). The BMI was lower in ESRD group vs. controls. Whereas inflammation markers (hsCRP and IL-6) and ADMA level were elevated in the ESRD group, total cholesterol was significantly lower in comparison with controls. There was no significant difference in blood pressure between the groups. Treatment with antihypertensive and lipid-lowering drugs can be explanation for the similarity in blood pressure between the groups, and for the lower total cholesterol level in ESRD patients compared to controls.

4.1.2 Resistance artery structure and function

The arteries from all groups had similar diameters, wall thickness, wall/lumen ratio and cross-sectional area indicating unchanged structure of resistance arteries in ESRD patients compared to controls (Table 2, Paper I).

4.1.2.1 Endothelial function

We show for the first time that flow-induced dilatation is impaired in the isolated resistance arteries from patients with ESRD (Figure 1A, Paper I). The presence of endothelial dysfunction in the resistance vasculature of ESRD subjects was further strengthened by a reduced dilatation in response to ACh (Figure 3A, Paper I). Current evidence of endothelial dysfunction in ESRD patients is primarily obtained from *in vivo* investigations on large vessels (Sahin and Yalcin et al., 2007; Verbeke and Agharazii et al., 2007) or measurements of circulating biomarkers (Stenvinkel et al., 2008). Although there was one study on the wire myography reporting reduced agonist-induced dilatation in subcutaneous arteries (Morris and McMurray et al., 2001).

We have shown the lack of NO contribution to flow-induced relaxation in ESRD patients (Figure 2A, Paper I). Our study suggests potential mechanisms behind small artery dysfunction in ESRD. Namely, the increased expression of nitrotyrosine in the vascular wall of arteries from ESRD patients may imply the enhancement of free radical production towards a pro-oxidant environment and NO degradation. On the other hand, the elevated circulating ADMA levels in ESRD patients might favour decreased NO production. Thus, reduced NO production and enhanced NO degradation are both involved in the altered NO availability in ESRD patients at the level of resistance arteries. The relative impact of each pathway to the general dysfunction, as well as possible alterations of other co-factors of NO synthesis, resulting in reduction of NO bioavailability remain unknown and deserve further investigation.

Our findings of blunted flow-induced dilatation and the lack of NO contribution to this response in human peripheral resistance vasculature extend and complement previous reports, but in other vascular beds (Annuk, 2005; Passauer and Pistrosch et al., 2005; Passauer and Pistrosch et al., 2005), emphasizing further the general state of endothelial dysfunction and the significant role of impaired NO availability in this group of patients with a high risk of CVD.

Moreover, we suggest that impaired shear stress-induced relaxation *ex vivo* might be more aggravated *in vivo* due to a high prevalence of anaemia in ESRD patients. Despite intensive erythropoietin treatment, ESRD patients have low haemoglobin levels (Table 1, Paper I) and, as a consequence, they experience low blood viscosity and reduced shear stress in comparison with healthy subjects. The evidence of abnormal shear stress value is suggested by *in vivo* studies (Samijo et al., 2002; Verbeke and Agharazii et al., 2007). Taken together, we postulate that in ESRD, the reduced ability of shear stress to induce relaxation, as presented in our study, along with anaemia-related low shear stress value *in vivo*, may further impede small artery wall ‘sensitivity’ to this physiological stimulus.

4.1.2.2 Contractile responses

We hypothesized that increased myogenic and agonists-induced constrictions might be contributing factors to the elevated peripheral resistance in ESRD. However, we found that the renal failure had no effect on contractile responses to changes in intraluminal pressure (myogenic constriction) and several agonists (AngII, ET-1, NE and phenylephrine) in isolated subcutaneous arteries (Figure 4A and Table 3, Paper I). These findings confirm preserved contractile vascular smooth muscle function in the resistance arteries of patients with ESRD. We assume that combined antihypertensive therapy could normalize the level of myogenic tone. This suggestion is supported by studies on animals (Osol G, 1986; Vettoretti S, 2006). Increased peripheral resistance in ESRD patients could be induced by elevated local concentrations of the tested vasoconstrictors, rather than changes in sensitivity of the arterial wall to them.

4.1.2.3 Distensibility

We found alterations in the passive properties of vascular wall (reduced distensibility index, Figure 4B, Paper I), that strengthens a link between ESRD and increased vascular stiffness reported before on large arteries (Blacher J, 1999; Peralta et al., 2009). Combining together our findings of changed distensibility and preserved arterial structure, we suggest that alterations in quality and/or quantity of vascular wall compounds might occur at the level of resistance arteries in ESRD patients.

4.1.2.4 Impact of cardiovascular co-morbidities

Exclusion of patients with diabetes and/or CVD had no impact on experimental outcomes (Figure 5, Paper I). This suggests that most likely uraemia itself, but not existing co-morbidities, is the main cause of endothelial dysfunction and altered passive properties of the resistance vasculature. Thus, our findings contribute to the elucidation of mechanistic links between uraemia and vascular abnormalities.

4.2 STUDIES II-III (PREECLAMPSIA)

4.2.1 Clinical data

Study II. The women with PE and healthy pregnancies were age matched. Nulliparous women comprised 40% and 52% in the case and control groups, respectively. Majority of PE women had preterm delivery with gestational age of 34 weeks (range 31–38) in the PE group vs. 38 weeks (range 37–40) in the control group.

Study III. There were no differences in age between the groups. Nulliparous women comprised 25% and 50% in the case and control groups, respectively. As expected, infant birth weight and the gestational age at delivery were significantly lower in women with PE (Table 1, Paper III).

4.2.2 Subcutaneous arteries (Study II)

In this study we failed to observe endothelial dysfunction in resistance arteries of women with PE. Indeed, the concentration-response curves of BK in PSS were similar between arteries from PE and normal pregnant women (Figure 4.1). Incubation of arteries with L-NAME plus Indo induced approximately 35-40% reduction in relaxation to BK in both groups compared to initial responses in PSS (Figure 4.1). This indicates that residual relaxation, which contains the main part of relaxation, is mediated by EDHF-type response. Since overall reduction of BK-induced relaxation after inhibition of NOS and COX was more pronounced in PE group vs. controls, we suggest that contribution of EDHF rather than NO is impaired in relaxation induced by BK in arteries from women with PE.

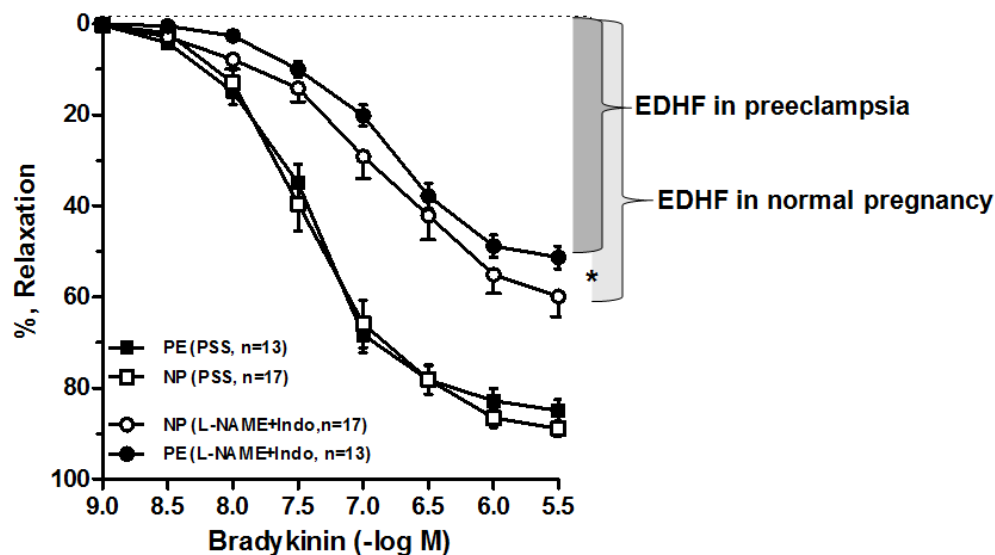


Figure 4.1. Concentration-response curves for bradykinin (BK) and contribution of EDHF in isolated subcutaneous arteries from normal pregnant (NP) and preeclamptic (PE) women in physiological salt solution (PSS) and after incubation with L-NAME in combination with indomethacin (L-NAME+Indo). * $P < 0.05$, PE vs. NP.

However, it should be stressed that unchanged contribution of NO to agonist-induced dilatation in isolated arteries could be affected by unfavorable preeclamptic conditions *in vivo*. The oxidative stress or elevated concentrations of endogenous inhibitor of NOS, ADMA, both of which are known to be observed in PE, might considerably reduce the NO bioavailability (Roggensack et al., 1999; Slaghekke et al., 2006; Poston et al., 2011).

4.2.2.1 Mechanisms of EDHF-mediated relaxation

It has been shown previously by our group that MEGJs were the major component of EDHF-mediated relaxation in subcutaneous resistance arteries in normal pregnancy (Luksha and Nisell et al., 2004). Identification of mechanisms involved in EDHF-type

responses in PE by incubation of arteries with inhibitors of pathways relevant to EDHF has revealed 1) an impairment of MEGJs pathway and 2) heterogeneity of EDHF contribution and its mechanisms.

Since inhibition of MEGJs revealed heterogeneity in EDHF-type response in arteries from women with PE, we divided the PE group into two subgroups (Figure 2, Paper II). In the PE-1 subgroup, incubation of arteries with 18- α GA reduced EDHF-type responses in similar way as in normal pregnancy, suggesting a main role of MEGJs (Figure 2A, Paper II). In the PE-2 subgroup, incubation of arteries with 18- α GA induced smaller but still significant reduction of EDHF-type relaxation in comparison with that obtained in normal pregnancy (Figure 2B, Paper II). This indicates that MEGJs did not play a sole role and some additional components were involved in EDHF-type relaxation in arteries from women in the PE-2 subgroup. Moreover, division into two subgroups revealed diverse overall contribution of EDHF to total BK-induced relaxation. In the PE-1 subgroup with a predominant role of MEGJs, EDHF-type response was impaired considerably. However, in the PE-2 subgroup the overall contribution of EDHF was preserved compared to controls.

Thus, we speculated that one or more additional mechanisms became involved in EDHF-type response in the PE-2 subgroup. They could serve to compensate the reduced MEGJs contribution and thereby preserve overall EDHF-type relaxation in PE to the level similar to that in normal pregnancy. Indeed, further investigation of EDHF-mediated mechanisms in arteries from the PE-2 subgroup supported our assumption. H₂O₂ and CYP450 metabolites of AA were responsible for EDHF-type relaxation in the PE-2 subgroup as supplementary or/and alternative candidates along with significantly reduced MEGJs component (Figure 4A and Figure 5A, Paper II).

For some unknown reasons the supplementary mechanism/s to MEGJs pathway were not triggered in the PE-1 subgroup and as a consequence the EDHF-type response became reduced in comparison with both normal pregnancy and the PE-2 subgroup. At this stage we cannot provide an explanation of heterogeneity in overall contribution and diverse mechanisms of EDHF-type response in subcutaneous arteries from women with PE and further research is warranted. Considering a heterogeneous character of PE as a pregnancy-related disorder, special attention should be paid to the selection criteria aiming to diminish this heterogeneity through the adjustment of recruited women according to the severity of the disorder. Such approach in the recruitment procedure is of importance especially in the research focused on identification of mechanisms behind of abnormalities specific for PE.

Implication of ultrastructural analysis of vascular wall supported our pharmacological evidence of MEGJs importance in subcutaneous arteries. In arteries from normal pregnant women, a relatively large number of long protrusions sent from ECs through the IEL toward SMCs were detected. ECs and SMCs formed close contacts (Figure 6, *F–J*, Paper II) previously referred to pentalaminar structures (Figuerola et al., 2004) and considered to serve as a prerequisite for MEGJs (Figure 6, *F–H*, Paper II). In contrast, in arteries from women with PE, we found a number of changes in morphology of the vascular wall. Those changes primarily included alterations in the morphology and size of ECs themselves (Figure 7, *C* and *D*, Paper II) accompanied by an apparent deficiency of projections toward SMCs (Figure 7, *B* and *E*, Paper II). The thickness of IEL had also a tendency to be enhanced compared to controls. Those morphological alterations could cause an obstacle to the establishment of tight intercellular contacts between ECs and SMCs (Figure 7, Paper II). Indeed, deficiency of pentalaminar-like

structures was observed in vessels from women with PE, resulting most likely in impaired communications between ECs and SMCs.

Thus, heterogeneous contribution of EDHF and involvement of MEGJs, H_2O_2 and CYP450 epoxygenase metabolites of AA in the EDHF-type responses were revealed in subcutaneous resistance arteries in PE. Such multifaceted changes in the mechanisms of endothelium-dependent relaxation could indicate that peripheral resistance arteries are indeed affected by the unfavorable conditions associated with PE. Therefore, we speculate that in spite of preserved overall endothelium-dependent relaxation to BK, the endothelial function of peripheral resistance arteries could be vulnerable and easily turned to the state of endothelial dysfunction *in vivo* and/or later in life.

4.2.3 Myometrial arteries (Study III)

In contrast to subcutaneous, in myometrial arteries from women with PE the overall endothelium-dependent response to BK (i.e. in PSS) was significantly reduced in comparison with normal pregnancy (Figure 4.2). Thus, endothelial dysfunction in PE, defined as reduced vasodilator response to endothelium-dependent agonist, was evident in the isolated small arteries from the uterine but not from the peripheral (subcutaneous) circulation.

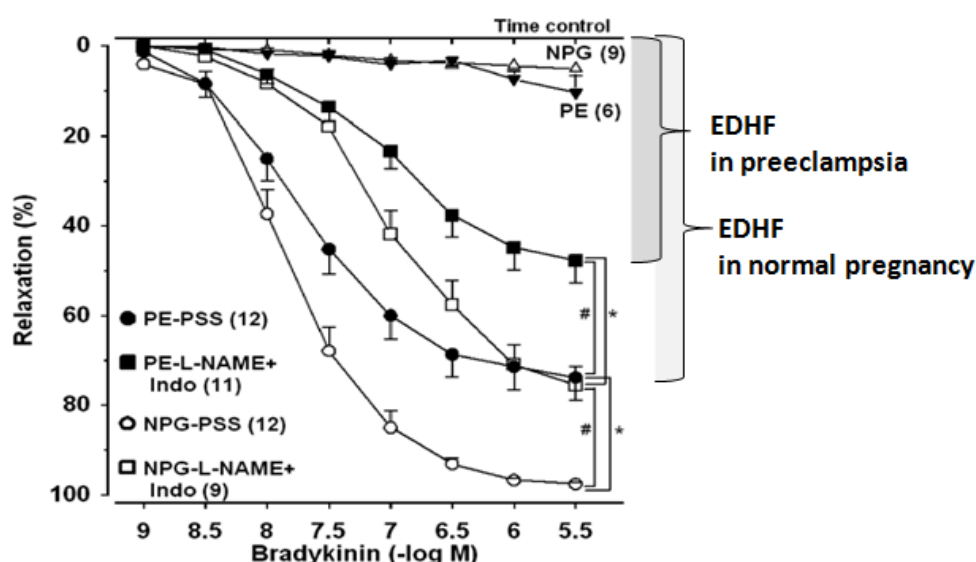


Figure 4.2. Concentration-response curves for bradykinin (BK), time control and contribution of EDHF in small myometrial arteries from normal pregnant (NPG) and preeclamptic (PE) women in physiological salt solution (PSS) and after incubation with L-NAME in combination with Indomethacin (L-NAME+Indo). $P < 0.05$; * - PE vs. NPG; # - before vs. after incubation with L-NAME+Indo. Open and closed triangles indicate a time control experiments only with the presence of vasoconstrictor.

As in subcutaneous arteries, incubation with L-NAME plus Indo resulted in significant reduction of BK-induced relaxation of myometrial arteries from both groups (PE and normal pregnancy) compared to that obtained in PSS. Moreover, the response to BK after incubation with L-NAME plus Indo was reduced in PE compared with normal pregnancy to the same extent observed in PSS (Figure 4.2). Hereby, we have concluded that in myometrial arteries 1) EDHF was a predominant mediator for endothelium-dependent relaxation to BK and 2) EDHF-type, rather than NO-mediated response was impaired in PE.

To clarify the mechanisms behind the reduced contribution of EDHF to endothelium-dependent dilatation in PE we applied diverse inhibitors of putative EDHF-mediated pathways by analogy with the Study II. As in subcutaneous, incubation of myometrial arteries from normal pregnant women with 18- α GA almost abolished the EDHF-type relaxation pointing out that MEGJs was a main component of EDHF-type responses of small arteries from both peripheral and uterine circulations (Figure 3, Paper III). Thus, our data are in line with previous finding for the potential role of MEGJs in EDHF-typed responses in myometrial arteries in normal pregnant women (Kenny and Baker et al., 2002).

In PE, pharmacological inhibition of MEGJs had no effect on EDHF-type relaxation in myometrial arteries (Figure 3, Paper III). This finding suggests that MEGJs pathway of EDHF-type relaxation was severely impaired in PE. To our knowledge, we for the first time showed that disturbances at the level of MEGJs could serve as an important contributory mechanism to endothelial dysfunction in PE in myometrial arteries. However, it is premature to speculate if MEGJ malfunction could play an underlying role in the genesis of vascular abnormalities and, eventually, clinical features of PE, although a potential role of Cx_s, which constitute MEGJs, for blood pressure control has been supported by experiments in Cx43 and Cx40 knockout mice (Liao Y, 2001; de Wit C, 2003).

To support our pharmacological findings about critical role of MEGJs in myometrial arteries we used the same ultrastructural analysis of the vascular wall, as in the Study II. Unfortunately, the analysis of TEM images was less efficient in the case of myometrial arteries isolated from both normal and PE pregnancies. In fact, we have rarely observed the contacts between ECs and SMCs, which could be referred as MEGJs. The discrepancy between pharmacological and morphological approaches to identify MEGJs in myometrial arteries has been previously reported by others (Sweeney et al., 2006; Kenny et al., 2002). In order to explain the difficulty to visualize MEGJs in TEM images, it has been suggested that most likely individual gap-junctional channels rather than plaques of gap-junctional channels are responsible for intercellular communication (Figueroa and Isakson et al., 2004). Moreover, extremely limited number of MEGJs and too low density of the gap-junctional channels might further aggravate visualization of MEGJs at the TEM images (de Wit et al., 2008).

Importantly, in spite of the lack of the MEGJ contribution in myometrial arteries in PE, the EDHF-type responses still comprised more than half of overall relaxation in response to BK (Figure 4.3). Thus, as in subcutaneous, in myometrial arteries from women with PE alternative EDHF pathways occurred. While administration of inhibitor of CYP2C9 epoxigenase revealed that AA metabolites did not participate in EDHF-mediated relaxation (Figure 5, Paper III), endogenous H₂O₂ had a substantial input into EDHF-type response (Figure 4A, Paper III). Thereby, an alternative EDHF pathway occurred predominantly via H₂O₂ in myometrial arteries in PE. Since catalase did not abolish EDHF-type response completely (Figure 4A, Paper III), the involvement of other mechanisms in EDHF-type responses in PE, including those yet unknown could be suggested.

Detection of H₂O₂ role in the control of endothelium-dependent relaxation of myometrial arteries in PE could partly explain the failure of the relatively recent “Vitamins in Preeclampsia-trial” (Poston et al., 2006). According to this trial, antioxidant vitamins supplementations from the second trimester of pregnancy in

women at increased risk for the gestational hypertension did not prevent PE, and increased the rate of low birth weight babies (Poston and Briley et al., 2006). Considering important role of H_2O_2 in the maintenance of vasodilator capacity of small myometrial arteries in PE, antioxidant therapy could possibly eliminate the dilative response induced by H_2O_2 . That could lead to decrease of diameter of myometrial arteries with further reduction in the blood supply to the fetoplacental unit and eventually could affect the fetal growth. However, further research is warranted to understand the detailed mechanism and contribution of H_2O_2 to the control of resistance arteries' tone in PE as well as the balance between beneficial and harmful roles of reactive oxidative species in the pathogenesis of PE in general.

4.3 STUDY IV (HISTORY OF PREECLAMPSIA)

4.3.1 Clinical data

The baseline clinical and biochemical characteristics of the women with a history of severe early-onset PE and women with a history of uncomplicated pregnancies are summarized in Table 1 and Table 2 in the Paper IV. Women were matched for age, BMI, smoking status and time after delivery. HELLP syndrome, recurrent PE, IUGR, and small for gestational age babies were the features of previously preeclamptic women at index pregnancy.

All women in our study were normotensive, non-obese and with normal glucose and lipids homeostasis. No evidence of kidney dysfunction and inflammation was detected in women with a history of PE. Thus, all women with a history of PE enrolled in our study were without known cardiovascular risk factors. However, there were significant differences in blood pressure, insulin level and HOMA index as well as a clear tendency ($p=0.05$) to the higher level of the waist to hip ratio in the case vs. control groups. Thereby, elevated levels of characteristics attributed to features of metabolic syndrome, found already 2 years after preeclamptic pregnancies in clinically healthy women suggest that these women may have a susceptibility to metabolic syndrome and they are likely to be predisposed for the development of hypertension and other cardiovascular complications later in life. Analysis of the clinical data of women at the first trimester of their index pregnancy, unfortunately did not give an answer if the features of metabolic syndrome were present before clinical manifestation of PE since the data were restricted ($n=11$) and had no measurements on lipid or precise glucose profile. Thus, it is still unclear if our findings of abnormalities at the level of resistance vasculature were induced by PE *per se* or they were inherent in these women and further unmasked when pregnancy advanced.

4.3.2 Resistance artery structure and function

This is the first study where structure, passive properties of vascular wall and endothelial and smooth muscle function of isolated resistance arteries were assessed in women with a history of severe early-onset PE. We found that structure of arteries was preserved (Table 3, Paper IV).

4.3.2.1 Dilative responses

Impaired endothelium-dependent dilatation to flow (Figure 1, Paper IV), but preserved response to BK (Figure 3, Paper IV) were found in women with a history of early-onset

PE. Our finding of reduced flow-induced dilatation in resistance arteries concurs with previous reports of the reduced response to flow in brachial artery *in vivo* in women with a history of early-onset PE (Germain et al., 2007; Hamad et al., 2007; Yinon and Kingdom et al., 2010). To our knowledge, we are the first who studied endothelium-dependent dilatation in response to BK in women with a history of PE. However, attenuated dilatation to another endothelium-dependent agonist ACh has been shown previously in the forearm blood flow and in the skin microcirculation 5-6 and 25 years after PE, respectively (Ramsay et al., 2003; Lampinen KH, 2006). The discrepancy could be attributed to different vascular beds, follow up periods or CVD risk profile. However, it is most likely that involvement of the specific receptors might be of importance, since in PE reduced endothelium-dependent responses to ACh but not to BK have been reported by others (Pascoal and Lindheimer et al., 1998).

Approximately equal contribution of NO to flow- and BK-induced dilatation was found in the control group (Figure 2A, Figure 3B, Paper IV). Interestingly, in the case group the same pattern of NO contribution was observed only in response to BK (Figure 3B, Paper IV), whereas complete absence of NO involvement was found in flow-induced dilatation (Figure 2B, Paper IV). These findings support the specific deficiency of NO contribution in response to flow as the most physiological stimulus of endothelium-dependent regulation of resistance artery function. It seems unlikely that enhanced NO degradation was the main reason for the lack of NO contribution to the flow-induced dilatation in the case group. Otherwise, equal inactivation of NO should occur irrespectively from the applied stimuli. Thereby, we speculate that disturbances of NO production may occur either via reduced sensitivity to shear stress (the same value of shear stress induced lower response in the case vs. control group) or impaired mechanism/s of eNOS activation by shear stress. It has been suggested, that the mechanisms of eNOS activation as well as sensitivity of eNOS to intracellular Ca^{2+} concentration might differ depending on stimulus applied (agonist or shear stress) (Fleming and Fisslthaler et al., 2001; Fleming, 2010).

4.3.2.2 *Contractile responses*

Our next important finding is increased myogenic tone in response to rapid increase in intraluminal pressure (Figure 6, Paper IV) and tendency to higher basal tone in resistance arteries from women experienced PE vs. controls. Since myogenic constriction is an important component of basal vascular tone, increased myogenic constriction might contribute to enhanced peripheral resistance and blood pressure. The tendency to the higher level of basal tone might contribute to higher blood pressure observed in the case group vs. control, even though all women were normotensive. It has been reported that previously preeclamptic women with higher mean and diastolic blood pressure had enhanced total vascular resistance vs. controls (Evans et al., 2011). Furthermore, the increased myogenic tone in response to rapid changes in intraluminal pressure indicates on activation of protective mechanisms against rapid increase of blood pressure in women with a history of PE. Thereby, in our study women with a history of PE could be already exposed to more frequent spontaneous variations in blood pressure. Indeed, the study on animals has shown that increased myogenic tone in mesenteric arteries was evident during the development of hypertension but not when hypertension was established (Izzard et al., 1996). Also increased myogenic tone but preserved structure of femoral arteries were reported in normotensive animals with mild experimental uraemia (Savage et al., 1998).

To our knowledge, we are the first who showed increased contractile reactivity to NE in isolated arteries from women with a history of early-onset PE vs. controls (Figure 8A, Paper IV). Thereby, we suggest that increased adrenergic receptors sensitivity and/or alterations in signal transduction pathways activated by adrenoreceptors stimulation might be responsible for enhanced contractile response to NE in resistance arteries from women with a history of PE. Since observed changes in adrenergic constriction took place in unison with alterations of myogenic constriction, it is possible to suggest that disturbances exist in general mechanisms of SMC constriction at the level of resistance arteries in women experienced PE. Indeed, NE- and pressure-induced vasoconstrictions utilize similar cellular transduction mechanisms (Davis and Hill, 1999). In contrast, no difference was found in the vascular response to Ang II between the groups (Figure 9, Paper IV). The differences in the molecular events of intracellular signaling between AngII-induced vs. NE- and pressure-induced constrictions (Kanaide et al., 2003) might be one of the reasons responsible for this discrepancy in contractile responses observed in our study.

4.3.2.3 Arterial distensibility

We found that isolated resistance arteries from women with a history of early-onset PE had reduced distensibility in comparison with controls (Figure 10A, Paper IV), indicating on increased stiffness or reduced elasticity of the vascular wall. Our finding is consistent with a recent report of reduced arterial elasticity of small as well as large arteries in women with a history of early-onset PE (Souwer and Blaauw et al., 2011). Other studies conducted on large arteries have reported contradictory results i.e. reduced or unchanged arterial compliance in previously preeclamptic women (Elvan-Taspinar et al., 2005; Rönnback and Lampinen et al., 2005; Spasojevic and Smith et al., 2005; Lampinen et al., 2006; Pàez and Alfie et al., 2009; Robb and Mills et al., 2009; Yinon and Kingdom et al., 2010). Currently the exact mechanism/s of reduced distensibility of resistance arteries from women with previous PE is open to speculation. It is difficult to declare whether changes in the composition and/or in properties of extracellular matrices components (i.e. collagen and elastin) exist.

4.3.2.4 Correlations

Despite the fact that, there was no difference in HDL, LDL/HDL ratio, triglycerides, HbA1c, hsCRP and homocysteine between the groups, we found significant correlations between mentioned above biochemical markers with some functional parameters within the case group. In fact, LDL/HDL ratio correlated positively with myogenic tone (max myogenic tone in response to rapid increase in intraluminal pressure, Figure 6B, Paper IV) and resting basal tone (Figure 7A, Paper IV). Moreover, resting basal tone was positively correlated with triglycerides, HbA1c, hsCRP and negatively correlated with HDL (Figure 7B-E, Paper IV). Finally, the negative correlation was found between homocysteine levels and distensibility (Figure 10B, Paper IV). At this stage it is premature to speculate whether cause-effect relationships exist within detected correlations. Based on our findings we can hypothesize that any subtle changes in the mentioned above biochemical parameters will be accompanied by worsening of certain functional properties. As a consequence, such events will lead to increase of peripheral resistance and progress to hypertension.

5 CONCLUSIONS:

The main findings presented in this thesis substantiate the functional alterations of resistance arteries in subjects with increased risk of CVD.

The principal conclusions are:

Study I: In patients with end-stage renal disease:

- The endothelium-dependent flow- and ACh-induced dilatations are impaired.
- The lack of NO contribution to flow-induced dilatation together with enhanced circulating levels of ADMA and enhanced nitrotyrosine staining in the vascular wall support the critical role of NO deficiency.
- Distensibility index is reduced, indicating on increased arterial stiffness.
- Observed alterations seem to be induced by renal failure *per se* rather than by CVD and/or diabetes mellitus.

Studies II-III: In pregnant women with preeclampsia:

- Reduced endothelium-dependent dilatation to BK is specific for myometrial arteries. This could contribute to the reduced uteroplacental blood flow.
- EDHF-type rather than NO-mediated responses are impaired in both myometrial and subcutaneous arteries.
- The contribution of MEGJs is reduced in subcutaneous and severely impaired in myometrial arteries.
- In subcutaneous arteries the contribution and mechanisms of EDHF-type relaxation are heterogeneous; MEGJs alone or in combination with either H₂O₂ or metabolites of AA comprise EDHF-type response.
- In myometrial arteries, the attenuated role of MEGJs is partly compensated through the contribution of H₂O₂ or other endothelium-derived factors.

Study IV: In women with a history of early-onset preeclampsia:

- Endothelium-dependent response to flow is impaired most due to the lack of NO contribution.
- Pressure-induced constriction is increased, indicating activation of myogenic regulation of vascular tone.
- Sensitivity to adrenergic receptors agonist NE is increased.
- Distensibility index is reduced, indicating on increased arterial stiffness.
- Although the blood pressure, insulin level and HOMA index are within the normal range, they are higher than in women with a history of uncomplicated pregnancies.
- A complex of functional alterations of resistance arteries, their correlations with some biochemical markers and changes in baseline clinical and biochemical characteristics emphasize the cardiovascular vulnerability of these women. Regular medical follow ups and promotion of healthy lifestyle are highly recommended.

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